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THE UNIVERSITY OF ALBERTA

THE ROLE OF CSF-1 IN IMMUNE INTERFERON-INDUCED DIFFERENTIATION OF THE MYELOID LEUKEMIA CELL LINE HL-60

BY

SIMEON VASSILIADIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (IMMUNOLOGY)

DEPARTMENT OF IMMUNOLOGY

EDMONTON, ALBERTA

SPRING, 1990

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ABSTRACT

This study revealed a previously unreported synergistic effect between colony-stimulating factor-1 (CSF-1) and immune interferon (IFN) that can induce human acute myeloid leukemia (HL-60) cells to complete differentiation and cease growth.

IFN induces some HL-60 cells to mature into monocytes. This action is apparent in the expression of functional CSF-1 receptors and changes in morphologic characteristics, is independent of at least tumor necrosis factor alpha (TNF) and interleukin-1 alpha (IL-1) and requires treatment for at least 48 hr. At 48 hr, there is a wave of transient IFN-mediated events, necessary for later up-regulation of CSF-1 receptors. The induced expression of CSF-1 receptors is pivotal in the differentiation program set in motion by IFN: through this receptor, CSF-1 transduces a mitogenic signal that promotes cell growth. However, this increased cellular proliferation is not followed by any discernible morphologic change. Although this situation persists for several cell cycles, after prolonged exposure to a mixture of CSF-1 and IFN the cells lose their proliferative potential and mature towards the myeloid pathway. Cell survival and normal myeloid morphology depend upon the continuous presence of IFN and CSF-1; when both factors are removed the cells die, whereas removal of either one results in increased cell accumulation.

The findings indicate the potential of CSF-1 to synergize with IFN to bring the M2 subclass of acute myeloid leukemia to clonal extinction.

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LIST OF ABBREVIATIONS

AML: Acute myeloid leukemia

CD 14: Cluster designation 14

c-*fms*: Cellular oncogene encoding the receptor to CSF-1

CM: Conditioned medium

CFU: Colony forming units

CSF-1: Colony-stimulating factor-1

CSF-1 receptor: Colony-stimulating factor-1 receptor

DMSO: Dimethylsulphoxide

G-CSF: Granulocyte colony-stimulating factor

G6PD: Glucose-6-phosphate dehydrogenase

GM-CSF: Granulocyte-macrophage colony-stimulating factor

FAB: French--American--British

FACS: Fluorescence activated cell sorter

HLA-DR: Human leukocyte antigen-DR

^{3}H-TdR: Tritiated thymidine

^{125}I-CSF-1: Iodine 125 labeled CSF-1

IFN: Immune (gamma) interferon

IL-1: Interleukin-1

IL-2: Interleukin-2

PBS: Phosphate buffered saline

TK: Tyrosine kinase

TNF: Tumor necrosis factor

TPA: 12-0-tetradecanoyl-phorbol-13-acetate

TPH: Theophylline

CHAPTER I

INTRODUCTION

Mature, functional blood cells are generated continuously from a common stem cell in a tightly regulated process of concomitant proliferation and differentiation from pluripotent stem cells to committed progenitors --> lineage-restricted precursors --> mature cells (Metcalf & Moore, 1971; van Furth & Cohn, 1978; van Furth et al., 1979). If this sequence of events is interrupted, immature myeloid blood cells accumulate and a disease state known as **myeloid leukemia** develops. The mechanism(s) by which the function of leukemic cells is controlled at the genetic or cellular level is still largely unknown. Studies *in vitro* have demonstrated that leukemic progenitors, like normal hemopoietic cells, depend upon **colony-stimulating factors** (CSF) for their proliferation and/or differentiation (Metcalf, 1986), but the heterogeneity of leukemic populations in general, has precluded elucidation of the role of CSF.

The present study examined how one CSF, **colony-stimulating factor-1** (CSF-1), affects differentiation of cells in one subtype of **acute myeloid leukemia** (AML). Because problems of population heterogeneity usually arise when primary (fresh) cells are employed, we developed, characterized, and used a cell line model that allows the study of the effect of CSF-1 on cellular leukemic proliferation and differentiation. This model uses the promyelocytic leukemia cell line **HL-60** (Collins et al., 1977), treated with

the lymphokine **gamma** or **immune interferon** (IFN; Inglot, 1983), to induce expression of functional CSF-1 receptors.

This introduction reviews the literature on normal and leukemic hemopoiesis, the cell line HL-60 model, and the reports of the role of CSF-1 in AML. It outlines the problem under study, and presents the working hypotheses, the specific objectives and rationale for the study, and the experimental approaches used to meet the objectives.

[A]. MYELOID LEUKEMIA

1. Clonal Origin of Myeloid Leukemia

It is well established that normal hemopoiesis is polyclonal, that is, many pluripotential stem cells contribute to blood cell formation (Fialkow, 1973). However, it is not known whether the same clones are maintained during the life span of an individual or whether individual clones sequentially express and become extinct. In contrast, chronic and acute myeloid leukemia (CML and AML) are hemopathies of a clonal origin (reviewed by Messner & Griffin, 1986). For CML, it originates in multipotential stem cells as demonstrated by two genetic markers: a) the Philadelphia chromosome (Ph'), and b) the glucose-6-phosphate dehydrogenase (G6PD) allotype (Fialkow et al., 1979; McCulloch & Till, 1977). For the former, chromosome 22 is characterized by loss of its long arm, which in most CML patients appears on chromosome 9. This cytogenetic hallmark, termed **t(9;22)** (Wintrobe, 1981; Nowell & Hungerford, 1987), results from translocation of the c-*abl* proto-oncogene from chromosome 9 to the *bcr* gene of no. 22, and reciprocal translocation of the c-*sis* proto-oncogene from chromosome 22 to no. 9 (McCaffrey et al., 1988). Although transcription of c-*sis* is not affected by its translocation, the head-to-tail juxtaposition of the 5'-end of c-*abl* with the truncated 3'-end of the *bcr* gene on chromosome 22 results in a transcriptional unit that produces an abberant *bcr-abl* fusion mRNA transcript. The product of this hybrid 8.5 kb mRNA and of the normal c-*abl* is a protein with tyrosine kinase (TK) activity; but their molecular weights differ, 210 Kd versus the normal 145 Kd (Korsmeyer et al., 1988). The Ph' chromosome also occurs in 20% of adults and 5% of children with acute

lymphocytic leukemia (ALL), and 2% of adults with AML (Kurzrock et al., 1988). In ALL, however, the translocation is at a different locus and the *bcr-abl* protein is of 185 Kd molecular weight, suggesting that different gene products may affect the outcome of the disease (Korsmeyer et al., 1988). The locus of the defect in AML has not been identified.

When the G6PD allotype is used as a clonal marker, G6PD heterozygotes test positive for type A or type B and only residual normal cells will have both enzyme types (Fialkow et al., 1979; Gartner & Kaplan, 1980). Therefore, using the G6PD system, one can determine not only the clonal origin of the leukemia but also its stem-cell lineage. For example, in a G6PD heterozygote with CML, circulating erythrocytes, platelets, eosinophils, monocytes and granulocytes all have a single enzyme type, whereas skin manifests both A and B types (Fialkow et al., 1977; Feviewed by Messnner & Griffin, 1986).

In AML, the blast cells that infiltrate the hemopoietic system are clonal and derive from a multipotential hemopoietic stem cell (Reviewed by Fearon et al., 1986). Isolated cases have been reported in which AML has been linked to a specific environmental agent such as X-rays (Court-Brown & Abbatt, 1955), occupational exposure to benzene (Aksoy et al., 1974), and therapy with alkylating agents (Rosner & Grunwald, 1980), but most cases are of unknown pathogenesis.

Cytogenetic, morphologic, and functional analyses led to the classification of the AML into many subtypes. According to the French--American--British classification (FAB; Bennett et al., 1976), there are eight types of AML: M1 (the most immature), M2, M3, M4, M5a, and M5b (the most highly differentiated), are myeloid in nature; and M6 and M7, representing erythroleukemias and megakaryocytoleukemias respectively.

The clonal origin of AML suggests that it is due to somatic heritable change(s) (Toksoz et al., 1989). Although there are non-random chromosomal abnormalities associated with nearly all cases of AML (Yunis et al., 1981), only one, is closely associated with an AML subtype; that is t(15;17) with M3 (Golomb et al., 1976). Thus, in contrast to other hemopoietic disorders with a shared chromosomal alteration, such as Burkitt's lymphoma with t(8;14) (Kaiser et al., 1977), follicular lymphoma with t(14;18) (Yunis et al., 1982), and CML with t(9;22) (Nowell & Hungerford, 1987), there is no karyotypic change in AML that is common to the disease as a whole.

Chromosome 5 seems to be of great importance in AML. Mapping studies have located several genes that encode growth factors and growth-factor receptors on this chromosome at 5q, including interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor receptor (PDGFR), beta$_2$-adrenergic receptor (B_2AR), endothelial-cell growth factor (ECGF), early-growth-response gene (EGR1), the monocytic differentiation surface antigen CD 14, and CSF-1, the CSF-1 receptor, and interleukins IL-4 and -5 (reviewed by Le Beau et al., 1989). Genes may be damaged or destroyed by deletion or by loss of an entire chromosome, thereby unmasking a recessive allele on the structurally normal homolog. A deletion may result in reduction of the gene product or loss of a wild-type allele. The clustering of genes encoding hemopoietic growth factors at the critical region of 5q suggests a role for these genes in the autocrine growth of myeloid leukemia cells characterized with a del(5q) (Le Beau et al., 1989; Oster et al., 1989).

Appropriate treatment of AML cells *in vitro* with biologic regulators

induces differentiation programs that help the cells to mature along the monocytic or granulocytic pathway (Collins et al., 1978).

[B]. NORMAL HEMOPOIESIS

The bone marrow, the principal source of blood cells, is one of the largest organs in the body. In normal adults, the net daily production of blood cells per Kg of body weight is about 2.5-billion erythrocytes, 2.5-billion platelets, and 0.1-billion granulocytes (Williams, 1984). The marrow is also the main producer of monocytes and lymphocytes.

The marrow contains the stem cells, a pool of cells dissimilar in morphology and function. Stem cells are capable of differentiation and self-renewal. The first report relevant to the characteristics of stem cells was published in 1961, with Till and McCulloch's observation of colonies of cells forming in the spleen of mice infused with syngeneic bone-marrow cells after destruction of their hemopoietic system by irradiation. These observations revealed that a single progenitor cell can give rise to a colony of cells, all of the same lineage, confirming Maximow's theory (1924) that a single cell is capable of giving rise to all mature blood cells.

The earliest marrow cell is pluripotential, capable of extensive (possibly life-long) self-renewal and differentiation to myeloid, erythroid, megakaryocytic and lymphoid progenitor cells. Next in line are the multipotential stem cells, (capable of self-renewal and differentiation to unipotential progenitor cells) that constitute the pools of cells committed to erythroid, megakaryocytic, and granulocytic lineages. An outline of the marrow-cell compartments is given in Figure I-1. Stem and progenitor cells are not very numerous, probably in a ratio of 1:1,000 to 1: 10,000 marrow cells. Their number may be influenced by the local micro-environment or regulatory factors that drive stem cells to develop into a more differentiated state (Metcalf & Moore, 1971; Metcalf, 1977).

Figure I-1

Lineage diagram of normal hemopoiesis.

1. Growth and Differentiation of Myeloid Progenitors

The blood cells derive from precursors known as stem cells (Sachs, 1986). Stem cells can multiply rapidly; normally, after maturing and differentiating into specialized forms, their progeny generally stop growing. Although the process is not fully understood, sufficient knowledge has accumulated about the regulation of growth and differentiation in normal cells that is now possible to answer several questions about abberant growth (malignancy) of cells.

The three phases of cellular growth in culture were defined by Hayflick and Moorhead (1961) in their studies with fibroblasts: phase 1, establishment of the primary culture; phase 2, a long period of proliferation during which the cells are outwardly normal; and phase 3, slowing of the growth rate, when cell morphology becomes abnormal, the yield of cells at confluence declines irreversibly, and finally the culture fails.

The regulation of differentiation remains one of the main unresolved issues in biology, a significant limitation to its study being the very nature of the process itself. In studying the control of gene expression, for example, in *E. coli* and other unicellular organisms, the investigator can realistically assume that the cell population is physiologically homogeneous and respond synchronously to a stimulus. In a multicellular organism, however, the nature of differentiation leads to heterogeneous cell populations, in which the biochemical events in individual cells differ in both substrate and timing and thus complicate the study of the populations (Levenson & Housman, 1981). Although it is fairly accepted that commitment to a particular cell type is the final cellular event leading to co-ordinated differentiation (Bell et al., 1978; Levenson & Housman, 1981), the stimulus that induces commitment is still speculative.

2. Commitment

The commitment of pluripotential stem cells to a certain line of development seems to be irreversible (Nicola et al., 1985) and is poorly understood. For example, the pluripotent stem cell is not responsive to **erythropoietin** (Epo) but at some point in development it gives rise to an Epo-responsive stem cell (Cline & Golde, 1979); such a cell is unipotent and exclusively committed to maturation along the erythroid pathway. An analogous situation holds for granulopoiesis and responsiveness to **colony stimulating activity** (CSA; Nicola et al., 1979). Thus, by extrapolation from these two reports, one may define commitment as the acquisition of responsiveness to a specific hemopoietin. The influence of specific factors induces further cellular proliferation and maturation until there are requisite numbers of functional end-stage cells (Cline &Golde, 1979).

3. Nongrowing state

What may distinguish a hemopoietic stem cell as normal is its spending periods in a non-cycling state, not passing through cell cycles continuously (Mauer & Fisher, 1962; Metcalf & Wiadrowski, 1966; Metcalf & Moore, 1971). Normally, restraint of cellular proliferative activity is accompanied by differentiation of these cells, but hemopoietic stem cells can retain and regularly exhibit the capacity to divide periodically. In many proliferative systems there is a balance between stimulation and inhibition (Williams, 1984); for example, it has been postulated that monocyte production is regulated by positive feedback of CSF and also by inhibitory factors elaborated by macrophages (Adams & Nathan, 1983; Johnson, 1978). In particular, prostaglandins of the E series (PGE) produced by macrophages are potent and specific inhibitors of monocyte generation *in vitro* (Zurier &

Sayadoff, 1975; Kurland & Bockman, 1978). Elaboration of PGE or other types of inhibitors by mature macrophages/cells may be modulated by CSF and thus affect the proliferation of monocyte progenitors and other myeloid cells. Products released by neutrophils, such as lactoferrin, are said to impair the release of CSF (Spry, 1983), however, the physiologic importance of lactoferrin as a modulator is uncertain (Williams, 1984).

[C]. THE LEUKEMIC HL-60 PROMYELOCYTIC CELL LINE

Heterogeneity of the primary cell population constitutes one of the commonest problems in studying human leukemia. In addition, primary cells cannot be propagated in liquid culture unless supplemented with nutrients and/or growth factors. Therefore, it is necessary to develop model systems that allow the study of normal and leukemic hemopoiesis.

The leukemic promyelocytic cell line HL-60 (Collins et al., 1977) used in this study represents an FAB-M2 subtype of AML (Dalton et al., 1988). The HL-60 cells grow autonomously, constitute a homogeneous population, and most importantly can be induced to express CSF-1 receptors (Sariban et al., 1987; Vassiliadis et al., 1989). As all AML subclasses express c-*fms*, the proto-oncogene that encodes the CSF-1 receptor (Dubreuil et al., 1988; Sherr et al., 1985), induced HL-60 cells are an excellent model for this study.

The HL-60 cell line was established in 1977 by Collins et al. from the peripheral blood of a woman with acute promyelocytic leukemia. However, the patient's blood showed cytogenetic abnormalities other than the typical t(15;17) and lacked several of the morphologic findings of the M3 type as initially classified (Collins et al., 1977; Gallagher et al., 1979). Since then, the HL-60 cell line has been reclassified as M2 (Dalton et al., 1988).

In AML, immature myeloblastic cells accumulate as a result of their slowed differentiation into nongrowing states. HL-60 cells are an extreme example of morphologically apparent limitation of differentiation and therefore are good models for studying reagents potentially able to control the pathophysiology of an immature cell's growth.

Phenotypically, most HL-60 cells appear to be arrested at the myeloblast and promyelocyte stage of differentiation (Collins et al., 1978;

Mannoni et al., 1982). The process of differentiation and development of HL-60 cells is influenced by several inducers of maturation (i.e., physiologic or chemical factors that help the cells to differentiate); therefore, the use of such agents allows one to study cellular events related to this process (Musson, 1983). Many regulatory compounds can be used to induce differentiation; those most commonly used to induce either granulocytic or monocytic differentiation are dimethylsulphoxide (DMSO), 1,25-dihydroxyvitamin D_3, retinoic acid, 12-0-tetradecanoyl-phorbol-13-acetate (TPA), sodium butyrate and interferons (Newburger et al., 1979; Carlson et al., 1984; Murao et al., 1985; Collins, 1987; Breitman et al., 1980; Sariban et al., 1987; Yourno et al., 1984; Boyd & Metcalf, 1984).

The growth pattern of HL-60 is autonomous. It is not known how this cell line propagates itself in liquid culture, and although Fibach et al. (1982) showed that it produces a substance which conditions the medium the action of this substance (CM) has not been established (reviewed by Collins, 1987). Olofsson and Olsson (1980) found that CM is of high molecular weight and termed it the "leukemia associated inhibitor", and stated that it prevents the entry of normal myeloid progenitors in S phase. Others claim that the HL-60 CM is an "autostimulatory activity" for this leukemic cell line (Perkins & Yunis, 1986).

Recent studies have characterized HL-60 at the level of genes and their expression, focusing in particular on the role oncogenes play in this leukemic line; e.g., both the N-*ras* and the c-*neu* genes are altered in HL-60, c-*myc* is stably amplified, the *p53* gene, at 17p13, is largely deleted, and one allele of the GM-CSF gene, at 5q21-q23, is truncated (reviewed by Dalton et al., 1988).

[D]. INTERFERON AS AN INDUCER OF DIFFERENTIATION

Alpha-, beta- and gamma-interferons are regarded as part of the nonspecific defense system against viruses, some micro-organisms, tumors and other abnormal cells. They can be defined as multifunctional substances whose main role is to control the proliferation of viruses and cells (Inglot, 1983; Stewart, 1977 & 1981). These glycoprotein molecules interact with their corresponding receptors (Langer & Pesta, 1988) on the outer surface of a cell's plasma membrane and trigger a cellular response.

Immune or gamma interferon (IFN) exists as one of two active species, both glycosylated and having molecular weights of 20,000 and 25,000. There is evidence of only one IFN gene, however, and the resolution of IFN into two components is due to post-translational processing of a 17-Kd protein. The IFN molecule has two glycosylation sites, at positions 28 and 100; the first is present in both 20- and 25-Kd molecules, and the second on only the 25-Kd species. The extent of glycosylation at each site is highly variable but even in its absence both species are biologically active. In humans, the DNA sequence of the IFN gene is on the long arm of chromosome 12; it contains three introns and a repetitive DNA element (Trinchieri & Perussia, 1985). This type of IFN, unlike the alpha- and beta-varieties (which bind to a different receptor), is produced by activated lymphocytes; it binds to a unique receptor, whose gene is located on chromosome 6q and has a molecular weight between 115 and 130 Kd (Aguet et al., 1988; Langer & Pestka, 1988).

One of the first effects ever described for IFN was its antiproliferative effect on various cell types, particularly on neoplastic cells (Rubin & Gupta, 1980; Raefsky et al., 1985). Its ability to restrict proliferation of HL-60 cells

makes IFN an excellent reagent for inducing HL-60 cells to differentiate to monocytes and for arresting the autonomous proliferation characteristic of malignancy. In addition, IFN can induce morphologic, histochemical, and functional changes in the HL-60 cells; i.e., the expression of antigens characteristic of monocytes (chapters III & V), and of cellular oncogenes (Sariban et al., 1987; Vassiliadis et al., 1989; Weber et al., 1989), the induction of alpha-napthyl acetate esterase, increased in cell size and decreased numbers of azurophilic granules (Ball et al., 1984).

The use of IFN as a mild, physiologic agent in therapy for AML has been well studied (Stewart, 1977 & 1981; Inglot, 1983). It was found that IFN is efficacious in some cases and also gives rise to few of the serious side-effects of most types of chemotherapy.

[E]. CSF-1 AS AN AGENT OF DIFFERENTIATION

The production of mature cells is regulated by several growth factors, and the development of precursors and proliferation of young macrophages is stimulated by three hemopoietic growth factors: IL-3, GM-CSF, and CSF-1 (Ihle et al., 1983; Metcalf, 1985 & 1986; Guilbert, 1985). The first two affect several differentiation lineages, whereas CSF-1 affects only the production of macrophages. Now it is known that IL-3 plays a dominant role in regulating the least mature cells, that GM-CSF dominates at intermediate stages, and CSF-1 at later stages (van Furth, 1985). However, all three factors stimulate the proliferation of end-stage murine macrophages, in varying degrees, and CSF-1 synergizes with either IL-3 or GM-CSF to form colonies from committed precursors in serum-free medium (Guilbert, 1985). These factors have been studied extensively in murine cells but their role in human cells is little known.

In AML, the prevalent concept is that the balance between proliferation and differentiation of myeloid precursor cells is disturbed, leading to their clonal expansion (Andreeff & Welte, 1989).

CSF may influence the behavior of human leukemic cells. Thus, GM-CSF induces the proliferation of certain leukemic cells without having an established degree of correlation at the level of the disease's subtype (Vellenga et al., 1987). GM-CSF acts directly on the cell cycle thereby influencing proliferation of the cells (London & McKearn, 1987). In one study, however, GM-CSF was unable to induce differentiation in all subclasses of AML unless in combination with other factors (Salem et al., 1989), although other investigators have reported some differentiation

abilities of GM-CSF that randomly induce leukemic cells to either the monocytic or the granulocytic pathway (reviewed by Andreeff & Welte, 1989).

Granulocyte colony-stimulating factor (G-CSF) induces both proliferation and differentiation of committed stem cells for granulocytes and monocytes, as assessed by clonogenic assays (Souza et al., 1986), and has potent effects on the M3 subclass of AML characterized by t(15;17) (Pèbusque et al., 1988). Coincidentally, the gene for G-CSF is located on chromosome 17 (Simmers et al., 1987).

It is believed that CSF-1 affects the differentiation rather than the proliferation of blast AML cells, a notion that has arisen from reports by Miyauchi et al. (1987 & 1988) that CSF-1 'selects' certain blast populations for differentiation. Complete evaluation of these results was not possible because of cell heterogeneity.

CSF-1 is a homodimeric glycoprotein produced by mesenchymal cells; it stimulates proliferation and enables mononuclear phagocytes to survive (Guilbert, 1985; Sherr et al., 1988). Two types of CSF-1 have been reported. The dominant mRNA species of 4.0 kb; it encodes a 554 amino acid (aa) polypeptide that contains an aminoterminal segment of 298 aa. Proteolysis occurs within this unique 298 aa segment; the released product has a relative weight of 86 Kd. The second form of CSF-1 originated from a DNA fragment of 1.6 kb, isolated from a phorbol ester-treated pancreatic cell line; it encodes a 256 aa polypeptide. The product of this DNA is synthesized as an integral transmembrane glycoprotein that is rapidly dimerized through disulfide bonds, externalized on the cell surface as a homodimer (68 Kd) and cleaved by proteolysis to generate a soluble extracellular growth factor (44 Kd) lacking transmembranous segments (Roussel et al., 1988; Rettenmier &

Roussel, 1988).

The use of colony-stimulating factors for clinical trials has been debated. However, the efficacy of these factors as therapeutic agents in AML is doubtful: 1) some CSF are potent stimulators of AML cell proliferation, which could enhance growth of the leukemic clone, and 2) their differentiation ability seems limited (Salem et al., 1989). It is widely believed that combinations of CSF with physiologic agents of differentiation could boost the induction of cell maturation. Such a mixture was used in this work --CSF-1 combined with IFN on the HL-60 cell line-- to examine this concept.

[F]. RATIONALE FOR THE STUDY AND DELINEATION OF THE PROBLEM

1. General objective

As documented above, studies of disregulation in the processes of differentiation/proliferation of AML cells are complicated by heterogeneous cell populations. Therefore, the main objective of this study was to develop a system for studying the control of the malignant proliferation of AML cells and more specifically the M2 subtype, which is characterized by excess myeloblasts and promyelocytes (Collins et al., 1977) unable to mature to a functional state.

The normal course of monocytic differentiation involves up-regulation of the CSF-1 receptor at about the monoblast stage (Guilbert, 1985). In the human system, very soon thereafter the cells cease growth (van Furth et al., 1979). It is not known whether specific ligand activation of the receptor results in this important differentiation step. AML is characterized by malfunction in the balance of growth and differentiation. Although AML subtypes can be associated with different maturation states in different lineages (Bennet et al., 1976; Collins, 1987), blast cell populations from all types express c-*fms* (Dubreuil et al., 1988). These observations argue that malfunctioning or perversely functioning CSF-1 receptor is involved in the AML phenotype. CSF-1 appears to both stimulate proliferation in AML populations and limit long-term population growth, effects attributed to the heterogeneity of primary populations (Miyauchi et al, 1988; Adreeff and Welte, 1989). How the CSF-1 receptor is expressed within AML populations is not clear: whether it is expressed functionally on all cells is also not known.

These problems of population heterogeneity and lack of knowledge of receptor distribution were important considerations in our choice of models. An AML population can be characterized as a separate lineage, comprising stem cell compartments able to self-replicate and increasingly more differentiated progeny. It differs from the normal counterpart in the major 'differentiated' product: AML blast cells. Although these blast cells typify the disease, their progenitors (and not the blasts) sustain it. Unknown is whether AML stem cells, blast progenitor cells or blast cells express c-*fms* or its functional product. Thus, it was not clear whether possible disregulation of CSF-1 signaling could occur by improper up-regulation of receptor, malfunctioning receptor or inappropriate signal reception by receptor-bearing cells. At the onset of this study, there was no way of separating primary AML populations into maturation stages for studies of the effects of CSF-1 (or lack of it). We therefore turned to a cell line model that allowed both controlled up-regulation of the CSF-1 receptor and examination of the effects of CSF-1 stimulation on up-regulated populations.

HL-60 cells, derived from an M2 AML patient (Collins et al., 1977; Dalton et al., 1988), express very low or no c-*fms* mRNA (Weber et al., 1989) but can be induced to express it by a variety of agents (see above). Of the agents considered, IFN induced a slow differentiation to growth competent monocytic-like cells. We therefore chose to develop this cell line model, framing our experimental approach around the following two working hypotheses.

2. Hypotheses

a) ***That IFN can induce HL-60 cells to express functional CSF-1 receptors during monocytic differentiation.***

b) That CSF-1 can act through its IFN-induced receptor to trigger a differentiation signal to initiate maturation, which can lead a leukemic clone to proliferative senescence and even extinction.

3. Specific objectives and experimental approaches

a) Characterization of the effect of IFN on HL-60 cells

Although much was known from the literature (see section C also) it was still necessary to quantify the capacity of IFN for induction of cellular differentiation and expression of the CSF-1 receptor. Morphologic studies, surface-marker analysis, and assays of function (e.g., phagocytosis and adherence) showed that IFN is unambiguously an inducer of monocytic differentiation, and this was confirmed by specific binding of the CSF-1 receptor and findings in autoradiography with ^{125}I-CSF-1 after induction. Assays of incorporation of tritiated thymidine (^{3}H-TdR) and autoradiography showed the fraction of cells in S phase, demonstrating that the CSF-1 had interacted through its receptor to promote a mitogenic signal.

b) Delineation of up-regulation of the CSF-1 receptor

Sensitive bioassays (chapter III) showed that the up-regulation of the receptor was not due to other cytokines produced by IFN. This was demonstrated by both ^{3}H-TdR assays and reduction of the tetrazolium salt MTT (which shows the metabolic activity of the cells in response to regulators of various types).

The time requirement for the IFN action was then investigated by using various induction protocols and examining receptor up-regulation as read-out.

Finally, cell-surface staining with specific monoclonal antibody and

FACS analyses demonstrated that up-regulation of the CSF-1 receptor after IFN treatment occurred only on a subpopulation of HL-60. This accounted for 35% of the total population.

c) The role of CSF-1 on CSF-1-receptor-positive HL-60 cells

When the characterization of the IFN-induced CSF-1 receptor was complete, we tried to isolate cells bearing CSF-1 receptors for the study of our second main hypothesis. Because detection of the CSF-1 receptor was only transient (detection was only possible on fixed cells), and sorting of this population was impossible, we used a HL-60 variant that constitutively expresses the receptor (C. Sherr, St Jude's Children's Hospital, Memphis, TN; personal communication). This sub-line was characterized with the methods used for the mother HL-60 line, and the action of CSF-1 in brief and long-term induced cultures was recorded. Although ^{3}H-TdR assays indicated a potential for CSF-1 as a differentiation agent, morphologic studies showed it had no effect on maturation. Also when combined with IFN, in some cultures the cells stopped growing and clonal extinction of a certain fraction of the leukemic clone occurred.

4. Reporting the results of the study

The work is reported as four papers (chapters II to V), with supplementary data in separate appendices.

Chapters II and III examine the effect of IFN on the monocytic differentiation of HL-60 cells, the induction of the CSF-1 receptor, and the kinetics of the receptor expression. Chapter III also examines whether other cytokines participate in the up-regulation of receptors, and rule this out, and clearly demonstrates the importance of IFN in the process.

Chapter IV reports IFN-mediated events observed during the first 48 hr after treatment, which indicated that up-regulation of receptors probably is triggered by some earlier event(s) not identified in this study. The importance of IFN as a differentiation agent is demonstrated: IFN appears to be one of the main biologic regulators in AML.

Finally, chapter V shows the effect of CSF-1 on cells bearing CSF-1 receptors and its ability to induce differentiation. Although CSF-1 displayed the potential to induce maturation, morphologic characterization of the cells contradicted this.

Investigation of the possibility that differentiation requires synergy of an unknown component with CSF-1 pointed to IFN: the combination of CSF-1 and IFN both induced morphologic differentiation and stopped the growth of the malignant cells.

Each section of this thesis is discussed individually. In addition, there is a brief general discussion at the end.

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CHAPTER II

APPEARANCE OF FUNCTIONAL IL-2 AND CSF-1 RECEPTORS ON HL-60 MYELOID LEUKEMIA CELLS AFTER INDUCTION OF MONOCYTIC DIFFERENTIATION BY GAMMA-INTERFERON.[1]

[A]. INTRODUCTION

Interferons, a group of glycoproteins exerting potent antiviral actions (Rubin & Gupta, 1980; Inglot, 1983), have a number of physiologically important effects outside this original definition. For instance, interferon gamma (IFN), a product of activated lymphocytes, differs from the other major species structurally and functionally (Trinchieri & Perussia, 1985) in participating in immunoregulatory functions (Nathan et al., 1983). Although IFN has been implicated as a suppressor molecule, most of its cellular effects are stimulatory: increased expression of MHC antigens (Sutherland et al., 1985; Ameglio et al., 1983; Rosa & Fellous, 1984 & 1988; Ball et al., 1984; Ucer et al., 1985) and other surface markers (Becker, 1984; Ball et al., 1984) on a variety of cell types, enhanced expression of the high-affinity Fc receptor for monomeric IgG (FcR1) on myelomonocytic cells (Trinchieri & Perussia, 1985), stimulated release of granulocyte macrophage-colony stimulating factor (GM-CSF) from HLA class II positive human monocytes (Piacibello et al., 1985), and the effects on HL-60 cells described below.

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In general, the effects of IFN on myelomonocytic cells involve activation of genes associated with differentiated monocytes (Finbloom et al., 1985; Ucer et al., 1985). We here examine the growth regulatory effects of IFN on myelomonocytic leukemia cells using the HL-60 line as a model (Collins et al., 1977). IFN slows the growth of HL-60 cells (Harris et al., 1985) and up-regulates the Tac antigen (one of the IL-2 receptor chains, Herrmann et al., 1985) and c-*fms* mRNA (which encodes the receptor to CSF-1, the monocyte-specific growth and differentiation factor, Sariban et al., 1987; Sherr et al., 1985). However, prolonged exposure (> 2 weeks) to IFN leads to gradual appearance of IFN-resistant clones which eventually overgrow the cultures (Vassiliadis, 1985). This communication asks whether a non-growing population of mature HL-60 cells exists after exposure to IFN, whether the Tac antigen mediates a mitogenic signal and whether *c-fms* mRNA is translated into functional CSF-1 receptor. We find that IFN initiates a program of monocytic differentiation in a subpopulation of HL-60 cells which involves a transient up-regulation of growth factor dependency and which may eventually lead to a normal non-growing state.

[B]. RESULTS AND DISCUSSION

Prolonged incubation (> 5 days) of HL-60 cells with IFN leads to two distinct populations of cells as assessed by intensity of HLA class I expression and volume analysis (Vassiliadis, 1985). In order to determine whether the two populations reflected different maturation states, they were sorted by FACS and examined for expression of the mature monocyte specific marker CD 14 with class I as a control (Table II-1).

The results indicate that the population expressing high levels of class I also expresses higher levels of CD 14 and therefore are the most mature. The ability of the sorted populations to proliferate, with and without the rich mixture of human hematopoietic growth factors contained in medium conditioned by 5637 cells (5637 CM), was then tested using a clonogenic assay (Table I-1). The more mature population, expressing high class I and CD 14, was found to contain very few clonogenic cells. Thus, IFN induces monocytic differentiation of a subpopulation of HL-60 cells to a non-growing state.

The IFN-induced up-regulation of cell surface Tac antigen and c-*fms* mRNA occurs only after a delay of 3 - 5 days. Therefore, in order to determine whether functional receptors were expressed, HL-60 cells were first incubated with IFN for 5 days, then subcultured and tested for clonogenicity in the presence of IL-2 (Table II-2), ability to specifically bind ^{125}I-CSF-1 on day 10 of culture (Table II-3-A) and growth rate between days 5 and 10 of culture with pure human recombinant CSF-1 (hrCSF-1, Table II-4). It was found that IL-2 partly reverses the IFN induced growth inhibition (Table II-2). The ability of anti-Tac antibody to totally block the effects of IL-2 indicated the reversal was mediated by the Tac antigen (anti-

TABLE II-1

Marker expression and proliferative potential of HL- 60 cells sorted for HLA class I expression after incubation with IFN.

A. Marker Expression*	Percent of cells positive after sorting into:	
Monoclonal antibodies specific to:	Low class I	High class I
---	0 - 10	0 - 10
83H1 (Class I)	10 - 30	60 - 100
82H3 (CD 14; monocytic)	0 - 10	30 - 60

B. Growth Potential+	Colonies (± SD) scored on day 10	
Cell stimulated by:	Low class I	High class I
---	20 ± 2	2 ± 0
5637 CM (10%)	76 ± 5	9 ± 1

* Cultured at 10^5 cells/ml with 100 U/ml IFN for 5 days, then sorted on a Coulter EPICS V after preparation for indirect immunofluorescence using 83H1 as primary antibody. The 82H3 antibody was used as a monocytic marker.

+ 5000 sorted cells/ml cultured in methylcellulose as described by Vassiliadis (1985).

TABLE II-2

Induction of functional IL-2 receptors on HL-60 cells after IFN treatment*.

Treatment	Colonies (±SD) scored on day 9	
	Untreated cells	IFN-gamma treated cells
---	368 ± 82	54 ± 10
hrIL-2 (100U/ml)	361 ± 57	183 ± 31
anti-Tac+hrIL-2	401 ± 103	50 ± 24
5637 CM (10%)	1532 ± 120	720 ± 86

* Cultures as described in legend to Table II-1. The cells were cultured in methylcellulose containing the indicated factors. Colony formation was detected after 7 to 8 days when aggregates of more than 48 cells were observed under an inverted microscope.

TABLE II-3

Number of ^{125}I-CSF-1 binding sites per cell after induction with IFN and/or theophylline (TPH)#.

Induction by:	Addition of CSF-1 on day 5	Sites/cell* (±SD)
A		
--	--	437 ± 54
--	+	<300
hrIFN-gamma	--	1618 ± 77
hrIFN-gamma	+	1347 ± 68
B		
TPH	--	<300
TPH	+	<300
TPH + hrIFN-gamma	--	<300
TPH + hrIFN-gamma	+	<300

0.18 mg/ml of TPH were added from the begining of the culture in order to inhibit the IFN-mediated up-regulation of the HLA-DR surface antigen.

* Calculated from net binding of human ^{125}I-CSF-1 ($2x10^5$cpm/ng) as described in Stanley and Guilbert (1981). Briefly, one hundred thousand cells were preincubated with 2 nM of CSF-1 at 4° and then ^{125}I-CSF-1 was added for the competition. The net binding was calculated and the number of binding sites per cell was estimated.

Tac antibody alone is somewhat stimulatory, 539±53 colonies versus 368±82 for the control). IFN up-regulates functional CSF-1 receptor between days 5 and 10 of culture both by criteria of specific binding of ^{125}I-CSF-1 (Table II-3-A) and mitogenic response to CSF-1 (Table II-4). For both up-regulation of binding sites and mitogenic response IFN, but not CSF-1 is required.
In contrast to IL-2, CSF-1 more than reverses the growth inhibitory effects of IFN (Table II-4). Identical results with CSF-1 were obtained using clonogenic assays (Appendix 1). Thus, IFN initiates a rather slow process which leads to up-regulation of functional receptors for both CSF-1 and IL-2.

The observation that up-regulation of functional CSF-1 receptors begins only after 5 - 8 days of exposure to IFN suggests a multi-step process. Expression of HLA class II antigen on HL-60 cells peaks after 2 days of culture with IFN (80% positive), then drops to background (15%) at day 8, just when CSF-1 receptor begins to appear. These kinetics suggest that transient expression of class II may be an intermediate step in the differentiation process. The Ca^{++}/calmodulin inhibitor Theophylline (TPH), among other actions, inhibits IFN induction of class II expression at day 2 of culture (Ina et al., 1987; Mauger et al., 1984; Hidaka et al., 1981). In order to determine whether class II expression was related to CSF-1 receptor appearance, the former was blocked at day 2 of culture with TPH and binding of ^{125}I-CSF-1 was assessed at day 10. TPH added to the cultures during the initial 5 day induction period with IFN blocked class II expression at day 2 (Appendix 2) and CSF-1 binding at day 10 (Table II-3-B). TPH added to the cultures between days 5 and 10 did not block binding (Appendix

TABLE II-4

The response of HL-60 cells to CSF-1 is regulated by IFN*.

		^{3}H-TdR incorporation (cpm ± SD)	
Five day induction by	Addition of CSF-1 on day 5	Day 8	Day 10
--	--	23565 ± 1225	39995 ± 103
hrCSF-1	--	25640 ± 440	36375 ± 402
hrCSF-1	+	26640 ± 1010	35320 ± 515
hrIFN-gamma	--	22655 ± 145	36550 ± 882
hrIFN-gamma	+	33335 ± 1275	49240 ± 1224
hrIFN + hrCSF-1	--	27435 ± 625	39080 ± 1029
hrIFN + hrCSF-1	+	32525 ± 425	45920 ± 1146

* 5 day induction carried out as described in Legend to Table II-1. On day 5, cells were subcultured at 5000/200ul/microwell. On the days indicated, cells were pulsed for 4 hrs with 1 uCi/well ^{3}H-TdR.

3) nor did TPH inhibit CSF-1 receptor expression on the human choriocarcinoma cell line JAR after a 24 hr incubation (824 $\pm$ 108 cpm with TPH versus 801 $\pm$ 79 in the control). These data show that TPH does not inhibit expression of CSF-1 receptors once upregulated and suggest that the same TPH inhibitable differentiation process initiated by IFN in the first 5 days of culture leads to transient class II, and late CSF-1 receptor, expression.

This study demonstrates that IFN induces HL-60 cells to: (a) differentiate ultimately to a non-growing state, (b) up-regulate functional receptors to IL-2 and CSF-1 and (c) initiate a differentiation process in which transient HLA class II expression is functionally related to later CSF-1 receptor appearance. Whether the transient expression of class II induced by IFN in leukemia cells is necessary for later appearance of CSF-1 receptor and whether subsequent activation of this receptor leads to complete monocytic differentiation (and possible clonal extinction) remains to be determined.

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CHAPTER III

INDUCTION OF FUNCTIONAL CSF-1 RECEPTORS ON HL-60 CELLS BY IMMUNE INTERFERON

[A]. INTRODUCTION

Immune interferon (IFN) is known to induce monocytic differentiation when used on the human promyelocytic leukemia cell line HL-60. The induction includes up-regulation of c-*fms* mRNA which encodes the receptor to the macrophage-specific growth factor colony-stimulating factor 1 (CSF-1). We here extend these observations to show that functional cell-surface receptor protein is expressed and that receptor-bearing cells are able to respond to mitogenic stimulation by CSF-1. We show that 33 % of IFN-treated HL-60 cells express high levels of receptors (approximately 6000 receptors/cell) and that CSF-1 increases ^{3}H-TdR uptake of IFN-treated cells by 60 to 100% after 10 days of culture. Up-regulation of CSF-1 receptors and response begins 8 days after initiation of IFN treatment yet requires only 2 days of IFN culture for full up-regulation. The presence of CSF-1 in the cultures during the up-regulation process does not alter the ability of IFN-treated cells to bind ^{125}I-CSF-1. Thus, after 48 hr of incubation with IFN, neither IFN nor CSF-1 is required or affects up-regulation of CSF-1 receptors. Data is also presented that strongly argues that two monokines that affect HL-60 differentiation, TNF alpha (TNF) and IL-1 alpha (IL-1), do not mediate the effects of IFN on up-regulation of CSF-1 receptors. Interestingly, TNF strongly stimulates expression of CSF-1 receptors on HL-

60 cells but does not co-ordinately up-regulate response to CSF-1. Taken together, these results show that IFN initiates a program of monocytic differentiation in HL-60 cells that proceeds with a 6-day lag period in the absence of IFN to culminate in up-regulation of functional CSF-1 receptors. The results also point to fundamentally different states of differentiation induced by TNF and IFN, one leading to CSF-1 receptor bearing cells mitogenically unresponsive to CSF-1 and the other to responsive cells.

Interferons (IFNs), a group of glycoproteins exerting potent antiviral actions (Rubin & Gupta, 1980; Inglot, 1983), have a number of physiologically important effects outside this original definition. For example, the aplha and beta types block mitogenic stimulation of quiescent Balb/c 3T3 fibroblasts by inhibiting the accumulation of mRNA that encodes the proto-oncogenes c-*myc* and c-*fos* normally observed during mitogenic activation (Greenberg & Ziff, 1984; Einot et al., 1985). In contrast, most of the cellular effects of IFN are stimulatory; e.g., they activate new genes, such as increased expression of MHC antigens (Ameglio et al., 1983; Ball et al., 1984; Rosa & Fellous, 1984; Sutherland et al., 1985) and other surface markers (Ball et al., 1984; Becker, 1984) on various cell types, enhance expression of the high-affinity Fc receptor for monomeric IgG (FcR1) on myelomonocytic cells (Trinchieri & Perussia, 1985), and induce the Tac antigen on HL-60 cells (Hermann et al., 1985; Vassiliadis et al., 1989).

The course of differentiation of normal human myeloid progenitor cells leads to proliferatively senescent neutrophils and macrophages (Pike & Robinson, 1970). These progenitors have an absolute dependency upon hematopoietic growth factors for survival, proliferation, and differentiation (Metcalf, 1986). The pathophysiology of myeloid leukemia involves accumulation of immature myelomonocytic cells that are a result of a slowed

rate of differentiation to nongrowing states (Till, 1982). The promyelocytic leukemia cell line HL-60 (Collins et al., 1977) is an extreme example of the apparent constriction in differentiation. These cells appear to be blocked at an immature and highly proliferative stage of differentiation and can be induced to differentiate along the granulocytic pathway by cis-retinoic acid and dimethyl sulfoxide (DMSO; Collins et al., 1978; Breitman et al., 1980), or the monocytic pathway by phorbol 12-myristate 13-acetate (PMA; Rovera et al., 1979), and IFN (Collins et al., 1978; Ameglio et al., 1983). In all instances induction of myeloid differentiation is accompanied by a reduction in growth rate (Rubin & Gupta, 1980). HL-60 cells are therefore good models for the study of reagents potentially able to bring the pathophysiology of leukemia cell growth under control (Collins et al., 1978; Rovera et al., 1979; Breitman et al., 1980). IFN is an excellent candidate reagent because its cytostatic effects induce a mild monocytic-like differentiation.

In this study we examine the effects of IFN on the appearance of responsiveness to CSF-1, the mononuclear phagocyte-specific growth and differentiation factor (Das et al., 1980). Biological response to CSF-1 is mediated by its receptor, a 160 Kd transmembrane protein found on cells of the monocytic differentiation lineage (Sherr, 1988) and encoded by the proto-oncogene c-*fms* (Sherr et al., 1985). HL-60 cells express low or undetectable levels of c-*fms* mRNA (Weber et al., 1989). Differentiation agents such as IFN up-regulate mRNA for c-*fms*, most likely post-transcriptionally (Sariban et al., 1985 & 1987; Weber et al., 1989) that predicts receptor protein appearance. Unknown is whether functional protein is induced and whether such expression leads to a response, either mitogenic or maturational. Therefore, in this report we ask whether and

when IFN up-regulates expression of functional cell surface CSF-1 receptor as assessed by high-affinity binding of ^{125}I-CSF-1 and appearance of CSF-1-stimulated growth. We find that IFN does up-regulate functional receptor by both these criteria, but only after a 6- to 8-day delay. IFN, however, is not required for receptor expression after an initial 2-day incubation period and the delayed up-regulation of CSF-1 receptor observed is not mediated by two other monokines, TNF and IL-1, also induced during monocytic differentiation (Trinchieri & Peroussia, 1986; Tsai & Gaffney, 1987).

[B]. MATERIALS AND METHODS

Cell culture: HL-60 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), grown in suspension culture in Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY) containing 2.5% FCS (Flow) at 37° C, in a humidified atmosphere containing 5% CO_2 in air.

Induction Protocol: One hundred thousand HL-60 cells were cultured with 100 U of IFN / ml for five days and then assayed for proliferation, expression of cell surface markers and specific binding of radiolabeled CSF-1.

Interferon: A preparation of human, recombinant IFN was a gift from Drs E. Falcoff and J. Wiezerbin (Institute Pierre et Marie Curie, Paris, France) and used at 100 U/ml.

IL-1 alpha: Pure recombinant human IL-1 was obtained from Dr P. Lomedico, Hoffman La Roche, Nutley, N.J. and used at a concentration of 1000 U/ml.

TNF alpha: Pure recombinant human TNF was purchased from Amgen Biologicals, Thousand Oaks, Ca. The concentration used ranged from 0.01 to 100 U or ng/ml.

CSF-1: Recombinant human CSF-1 was obtained from Dr P. Ralph, Cetus Corp., Emeryville, Ca., and was used as an unpurified supernatant from CHO cells plasmid-transfected using the truncated form cDNA of Kawasaki et al. (1985). This factor was used at a concentration of 1300 U/ml.

^{125}I-CSF-1 binding assay: CSF-1 was labeled with ^{125}I using a modified chloramine T method to achieve a specific activity of about 10^{18} cpm/mol of protein (Stanley & Guilbert, 1980). The iodinated preparation was routinely

tested for bioactivity on growth factor dependent, murine macrophage cell lines (Branch & Guilbert, 1989).

Cells were cultured in CSF-1-free medium one day before the binding experiments to upregulate the CSF-1 receptor density and then washed twice with cold phasphate buffered saline (PBS) and resuspended at 150,000 cells per ml after a 20-minute preincubation period on ice in ImB (IMDM minus bicarbonate tittered to pH 7.35 with NaOH) in preparation for the binding reaction. Binding was carried out in 15-ml polypropylene tubes (Corning) at a final volume of 200 to 250 microliters at 4° C. Specific binding was determined as the amount of ^{125}I-CSF-1 binding that could be blocked by a one hour preincubation with 2 nM of unlabeled CSF-1 (Stanley & Guilbert, 1980; Guilbert & Stanley, 1986). The binding reaction was stopped and unbound label separated from bound by centrifuging the cell suspension through an ice-cold FCS layer. The ^{125}I content of the pellet was determined in a LKB-Wallac CliniGamma 1272 rack counter (Finland).

Because CSF-1 forms an unstable complex at 4° C, when it binds to its receptor (there is no K_{off}), Scatchard analysis cannot be performed for the determination of the binding sites. Therefore, the number of receptors per cell was determined by the formula:

$$\text{Receptors/cell} = N \frac{\text{Bound (cpm)}}{\text{S.A x Number of cells employed}}$$

where $N = 6.02 \times 10^{23}$ and S.A = specific activity.

All binding experiments shown here were carried out with preparations having specific activity of $1.0x10^{18}$ cpm/mol. Saturation of all available sites was confirmed by demonstrating that the excess amount of label added in various experiments corresponds to plateau binding levels.

Tritiated Thymidine (^{3}H-TdR) Uptake: Cell cultures were maintained in triplicate wells of flat-bottomed microculture plates (Linbro, Flow Laboratories, Hamden, CT). Five thousand cells in a final volume of 200 ul were incubated at 37° C in 5% CO_2 in air for up to 7 days. Cultures were pulsed for 4 hrs with one uCi/well of ^{3}H-TdR (New England Nuclear, Boston) prior to harvesting and counted on a Packard Minaxi B 4000 liquid scintillation counter (Packard Instruments, Downers Grove, IL). ^{3}H-TdR uptake is expressed as mean counts per minute (cpm) $\pm$ standard deviation (SD).

Autoradiography: After the cells were pulsed with ^{3}H-TdR for 18 hr or incubated with ^{125}I-CSF-1, they were washed once with ice-cold PBS, followed by a centrifugation through an ice-cold FCS layer and a second washing with cold PBS. Then the cells were cytocentrifuged onto a slide and fixed in ice-cold methanol for 20 minutes. The slides were dipped in warm (42° C) emulsion (Kodak NTB2, Eastman Kodak Co., Rochester, N.Y.), dried and enclosed in light-excluding containers for 4 to 20 days at 4° C. After warming at 22° C the slides were developed for 2 minutes in Kodak D-10 developer, rinsed for 30 seconds in 1% acetic acid, fixed for 3 minutes in Kodak fixer, and then washed in five changes of distilled water.

Positive cells were identified by a dark grainy nucleus for the ^{3}H-TdR experiments, whereas ^{125}I-CSF-1 autoradiograms were scored positive when the grains were at the periphery of the cells.

Phagocytosis: Phagocytosis was assessed by incubating the cells for 30 to 45 minutes with 0.57 u $\emptyset$ fluorescent beads (Polysc. Inc. Warrington, PA), washing extensively and reading under a fluorescent microscope. Cells were scored as phagocytic when more than 10 beads were seen in the cytoplasm.

Colony formation assay in methylcellulose: Five to ten thousand HL-60 cells per ml were plated in methocellulose (final methocel concentration 0.8% w/w) in 35 mm culture dishes (Falcon). Each dish contained the following ingredients: the cells under test immobilized in the viscous culture medium (methylcellulose plus alpha-MEM), 5% FCS, and where necessary IFN and CSF-1. All the components were added to a Falcon plastic test tube in a total volume of 3 mls. This was mixed well by vortexing and the suspension obtained was dispensed in one-ml aliquots into Falcon plastic petri dishes using a syringe with a 18 $^{1/2}$ G needle. The cultures were incubated at 37° C for 8 days in 5% CO_2 in air and the colonies (aggregates of more than 48 cells) were scored using an Olympus inverted microscope.

MTT assay/TNF bioassay : The bioactivity of TNF in the HL-60 supernatants was assessed by specific killing of L929 cells as monitored by reduction of the tetrazolium salt, MTT (Branch et al., submitted). TNF-resistant and -sensitive cells (L929-RFEB and L929-8 respectively) were plated on day 0 at a concentration of $4x10^4$ / 50 microliters in 96-well dishes (Linbro) and incubated at 37° degrees for 24 hr in the presence of IMDM containing 5% FBS and 0.5 ug/ml mitomycin C. On day 1, human TNF standards (100 U/ml to 0.5 U/ml) and the supernatants under test were added in triplicate at a final volume of 50 ul. After two days of incubation (day 3), the metabolic activity of the cells was estimated by their ability to reduce MTT (0.5 mg/ml) when incubated for 4 hr. Acid-isopropanol was added to all wells and the plates were mixed on a shaker for 5 minutes to

dissolve the insoluble blue reduction product. The individual wells were then read on a Dynatech MR600 microplate reader using a test wavelength of 560 nm and a reference wavelength of 630 nm. The sensitivity limit of the assay was about 30 pg/ ml.

IL-1 bioassay: IL-1 activities in HL-60 supernatants were quantitated as essentially described by Gearing et al., (1987), Briefly, supernatants were tested on a co-culture of the murine cell lines NOB-1 (EL4-6.1 cells, unable to incorporate thymidine since they are tk^-, IL-1 responders and IL-2 producers) and CTLL cells (IL-2 responders). The assay measures the incorporation of ^{3}H-TdR (6 hour pulse) by CTLL cells to IL-2 produced by the NOB-1 population in response to IL-1 present in the supernatant(s) during a 24 hour culture. Fifty thousand NOB-1 cells and ten thousand CTLL cells were co-cultured at a final volume of 100 ul in a 96-well dish (Linbro). Control wells contained CTLL cells alone. No response was obtained with CSF-1, GM-CSF, IL-3, TNF, LPS and LIF. Mouse and human IL-2, as well as mouse IL-4, directly stimulate CTLL growth and thus could mask any IL-1 effects.

Flow cytometric analysis: The indirect immunofluorescent analysis of cell surface antigen expression was performed as follows. Cells were incubated for one hour at 4° C with the appropriate antibody (83H1 a mouse IgG1, class I; 7H3 a mouse IgG2a, class II; 82H3 a mouse IgG2a, CD14; 80H3 a mouse IgG2a, CD16; MacLean et al., 1982; Mannoni et al., 1982 and 33.B31 a CD25 rat IgG2a; light chain of IL-2 receptor, a gift from C. Mawas, France) then washed three times with cold PBS and incubated for another hour at the same conditions with FITC-conjugated goat anti-mouse IgM, IgG and IgE antibody (Cappel Lab., Cochranville, PA). After washing three

times, the cells were fixed in 2% formaldehyde and the fluorescence intensity of individual cells quantified by flow cytometry using an EPICS V fluorocytometer equipped with a 5-watt argon laser (Coulter Electronics, Hieleah, FL).

As controls we used a number of monoclonal antibodies specific for T cells having the same isotype. For the murine IgG1 we used the B9.11 (CD8) antibody, for murine IgG2a, the CD7 and CD5 88H1 and BL1 antibodies respectively and finally for the rat IgG2a we used the CD2 antibody known also as 39.C15. These antibodies were obtained from Dr. P. Mannoni (Immunotech, Marseille, France).

[C]. RESULTS

The effects of IFN on HL-60 growth and differentiation.

Treatment of HL-60 cells with IFN induces differentiation as assessed by: (a) gross morphology, phagocytosis and cell surface antigen expression (Ball et al., 1984a & 1984b; Becker, 1984 and Table III-1; non-binding antibodies of same isotype were used as controls: see footnote of Table III-1); (b) expression of the receptor for IL-2 (the Tac antigen, Herrman et al., 1985; Vassiliadis et al., 1989) and for mRNA encoding TNF and the receptor for the monocyte specific growth and differentiation factor CSF-1 (c-*fms*; Sariban et al., 1985 & 1987); and (c) a decrease (13%) in the rate of growth of the population and a much lower decrease (65%) in the number of colony forming cells in it (Table III-2). Prolonged culture with IFN alone did not lead to an irreversible cessation of growth because by two weeks of culture clones of IFN-resistant cells appeared at increasing frequency (Vassiliadis, 1985). While the above characteristics are all typical of a differentiation process, only morphology, expression of CD-14 antigen (Goyert et al., 1988; Simmons et al., 1989) and c-*fms* mRNA specify monocytic differentiation. These data indicate that IFN stimulates monocytic differentiation of HL-60 cells, albeit in a partial and apparently asynchronous fashion. Accumulation of c-*fms* message began after 7 days (Sariban et al., 1987). After 5 days of IFN treatment, 40% of the treated cells acquired monocytic morphology and were positive for CD- 14 expression (Table III-1).

Examined below is whether expression of c-*fms* mRNA results in expression of functional CSF-1 receptor and to what degree this might occur.

TABLE III-1

IFN-induced monocytic differentiation of HL-60 cells.

A. Percent of cells able to phagocytose 0.57 u fluorescent beads*.

Day	-IFN	+IFN
4	2 ± 1	17 ± 3
5	4 ± 1	23 ± 4
6	4 ± 2	37 ± 5
7	5 ± 2	47 ± 5

B. Percent of monocyte / macrophage cells by morphology*.

5	15 ± 2	40 ± 2

C. Percent of positive cells by flow cytometric analysis* at day 5.

Antibody (specificity)	-IFN	+IFN
PBS	08	10
83H1 (class I)	86	98/(9)@
7H3 (class II)	17	40/(6)
82H3 (CD 14 monocytic)	15	42/(6)
80H3 (CD 16 myelocytic)	42	62/(10)
33.B3 (CD25 IL-2 receptor)	04	20/(7)

* Phagocytosis, morphology and flow cytometric analysis of cell surface marker expression carried out as described in the Methods.

@ Number in parenthesis indicates the percentage of cells that expressed T cell markers of same isotype (see Methods).

Up-regulation of functional CSF-1 receptor

Function was examined in two ways: (1) ability to specifically bind biologically active ^{125}I-CSF-1 at high affinity and (2) ability to transduce a mitogenic signal at physiologic CSF-1 levels. HL-60 cells were cultured with IFN (100 U/ml, a level found to induce near maximal differentiation, Vassiliadis, 1985) for 5 days, then counted and subcultured with or without fresh IFN and with or without CSF-1 (1300 U/ml) for up to 9 more days. The ability of the population to specifically bind ^{125}I-CSF-1 and to incorporate ^{3}H-TdR in response to CSF-1 was assessed at the end of the subculture (days 6-14 of culture). Expressed as the average number of ^{125}I-CSF-1 molecules bound per cell at 4° C in the total population, binding sites did not appear until after the initial 5-day induction with IFN (Table III-3). Thereafter, the number steadily increased as a function of time. The fraction of cells binding ^{125}I-CSF-1 on day 10, determined by autoradiography, was approximately 33 $\pm$ 3 % (Fig. III-1). In agreement with this slow appearance of receptor, CSF-1 did not stimulate proliferation significantly until day 8 of culture (Table III-2). The ^{3}H-TdR labeling index of cells cultured with CSF-1 was 36% higher at day 8 and almost 70% higher at day 10 than that of IFN-treated cells cultured without CSF-1 (Table III-2). Even relative to rapidly growing untreated controls, cells cultured with the combination of CSF-1 and IFN grew more rapidly. In order to determine the fraction of IFN-induced cells that respond to CSF-1, cells taking up ^{3}H-TdR over an extended (18 hour) pulse time with and without CSF-1 were compared by autoradiography (Table III-4). The fraction of cells stimulated by CSF-1 (37% on day 10) nearly matched the fraction of cells able to specifically bind ^{125}I-CSF-1 on the

TABLE III-2

CSF-1-stimulated proliferation of IFN-treated HL-60 cells@.

A

Culture components		^{3}H-TdR incorporation (cpm ± SD) on days*:	
CSF-1	IFN	8	10
--	--	27936 ± 839	69653 ± 823
+	--	28588 ± 135	70570 ± 1027
--	+	22130 ± 519	60444 ± 782
+	+	30712 ± 654	102774 ± 3260

B

	Colonies per ml of culture#	
CSF-1(U/ml)	- IFN	+ IFN
--	306 ± 30	108 ± 16
+(1000)	290 ± 45	325 ± 55
+(100)	225 ± 28	340 ± 26

@ The ^{3}H-TdR and clonogenic assays were performed as described in the Methods.

* n=10; in triplicate cultures

n=12; in triplicates

TABLE III-3

Net CSF-1 receptor numbers on HL-60 cells with and without IFN treatment.

	Net number of receptors / cell* (±SD)	
Day of culture	- IFN	+ IFN@
5	<50	<50
9	307±42	656± 99
12	156±70	1,529±187
14	204±65	2,076±310

* Calculated as noted in the methods, using a labeled preparation with a specific activity of 1.5×10^{18} cpm/mol.

@ Cells were cultured with IFN for 5 days, and the binding assay was performed on the days indicated above.

TABLE III-4

Percent of cells in S phase of their cell cycle after treatment with IFN and CSF-1 as assesed by autoradiography.

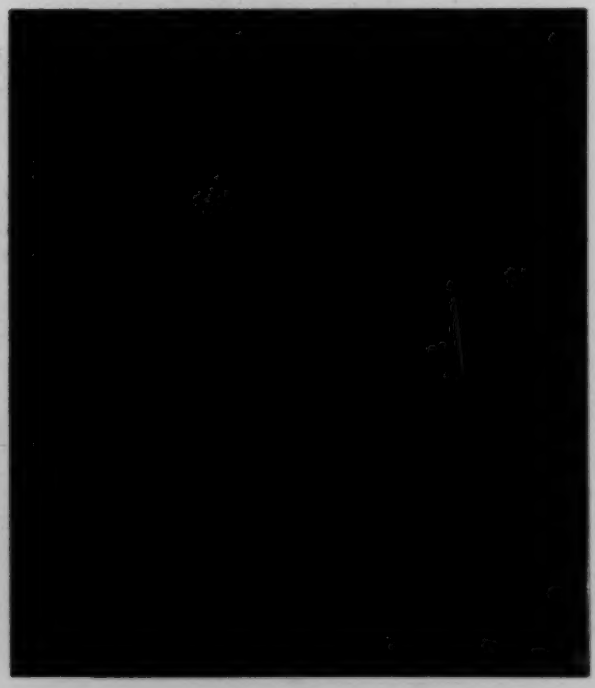
Culture components		% of cells incorporating ^{3}H-TdR ($\pm$SD)	
CSF-1	IFN	Day 8	Day 10
--	--	52 $\pm$ 5	50 $\pm$ 3
+	--	50 $\pm$ 6	49 $\pm$ 11
--	+	32 $\pm$ 7	33 $\pm$ 4
+	+	68 $\pm$ 4	70 $\pm$ 5

The cells were pulsed on the indicated day with ^{3}H-TdR for 18 hr before being processed for autoradiography as described in the methods.

Figure III-1

Autoradiographic study demonstrating HL-60 cells to bind ^{125}I-CSF-1. The cells were processed as described in the methods. The percentage of cells positive was calculated from the autoradiographic slides and was as follows:

Culture conditions	Percentage of cells positive for the receptor (±SD)
Untreated cells	9 ± 3
IFN	33 ± 3



same day as determined by ^{125}I-CSF-1 autoradiography (33±3%). Thus, IFN up-regulated functional receptors by two criteria: binding of ^{125}I-CSF-1 and response to CSF-1.

Extracellular requirements for induction of functional CSF-1 receptors by IFN

Expression of mRNA encoding TNF and IL-1 has been shown to be induced in monocytes by IFN (Fibbe et al., 1988 & 1989; Sariban et al., 1985; Newton, 1985; Tsai & Gaffney, 1987). TNF has also been shown to have potent synergistic effects on the differentiation of HL-60 cells to monocytes when combined with IFN (Trinchieri et al., 1986) and in promoting proliferation of murine bone marrow-derived macrophages when used in the presence of CSF-1 (Branch et al., 1989). Thus, the possibility that either cytokine could mediate the effects of IFN during the induction period was examined. HL-60 cells were cultured with and without CSF-1, IL-1 and TNF over a 10-day period (Table III-5). The data show that although TNF inhibited growth and IL-1 somewhat stimulated growth, neither cytokine increased the responsiveness of HL-60 cells to CSF-1 in the absence of IFN. These results indicate that the IFN-induced up-regulation of responsiveness to CSF-1 is not mediated by release of IL-1 or TNF but do not exclude the possibility that low levels of released cytokines might synergize with IFN. This possibility is examined in more detail below.

Assay of supernatants from IFN-treated cultures on TNF-sensitive and -resistant cell lines as described in the Methods revealed no detectable (up to 50 pg/ml) TNF released from 10^5 cells/ml in 5 days of culture (Appendix 4). Titration of the inhibitory effect of TNF on HL-60 growth

TABLE III-5

Effect of IL-1 alpha and TNF alpha on the growth of HL-60 cells.

Addition to culture*	^{3}H-TdR incorporation (cpm ± SD) Day 4	Day 8	Day 10
nothing	14060 ± 604	43790 ± 2310	69470 ± 530
CSF-1	14488 ± 142	45055 ± 2415	70770 ± 830
IL-1 alpha	16334 ± 855	73575 ± 1725	77670 ± 3503
TNF alpha	20021 ± 107	33081 ± 983	30848 ± 1104
CSF-1+IL-1	16649 ± 329	72685 ± 405	78860 ± 1318
CSF-1+TNF	22553 ± 996	32849 ± 1415	30339 ± 949

* HL-60 cells were cultured as described in the Methods. The cells in this experiment were not induced with IFN. The factors were added at the beginning of the culture: IL-1 was present at 1000U/ml, TNF at 10 U/ml (on day 10, 50 U/ml gave results identical to 10 U/ml), and CSF-1 at 1000 U/ml. ^{3}H-TdR uptake was determined (n=4; in triplicates) on the days indicated.

showed a smooth dose-dependent effect which was maximal at 25 ng/ml at approximately 75 % inhibition, half maximal at 1 ng/ml and was still significant at 70 pg/ml (Table III-6). In order to determine whether this inhibition of growth , like that induced by IFN, was accompanied by differentiation into a CSF-1 receptor-bearing state, we asked whether CSF-1 receptors were coordinately up-regulated. The results in Table III-7 show that TNF at 2 ng/ml induced appearance of approximately the same level of specific high-affinity CSF-1 binding after 5 days of incubation as did IFN after 10 days, that this level of CSF-1 binding persisted at least to 10 days of culture, and that IFN at 100 U/ml did not significantly increase or decrease the level of TNF-induced binding on day 10. Thus, TNF up-regulates CSF-1 receptors (Table III-7) but does not induce responsiveness to CSF-1 (Table III-5).

We next determined whether low levels of TNF (0.5 ng/ml) might synergize with IFN to induce responsiveness to CSF-1 (Table III-8). The results indicate that TNF inhibited HL-60 growth under all growth conditions and specifically did not co-operate with IFN at any concentration to induce up-regulation of responsiveness to CSF-1 even though both cytokines up-regulate receptors equally well. In order to rule out production of very low levels of TNF involved in the CSF-1 response obtained, we treated HL-60 cells with IFN for two days (see below) and then washed the cells a number of times. Subsequently, CSF-1 was added to the cultures and responsiveness was monitored by ^{3}H-TdR uptake. We found that the washed cells could respond and bind CSF-1 on day 10 suggesting that a possible low level production and accumulation of TNF due to IFN was not involved (Appendix 5). Taken together the above results strongly argue that even if IFN induces

TABLE III-6

Dose response of TNF on HL-60 cells and percentage of suppression*.

Treatment	^{3}H-TdR incorporation (cpm ± SD) on day 5	% Suppression
None	66159 ± 1012	0
100U/ml TNF	15377 ± 245	76.75
50	20021 ± 788	69.70
25	16933 ± 379	74.40
05	35502 ± 1819	46.30
2.5	43143 ± 639	34.80
1.25	40277 ± 215	39.10
0.6	43866 ± 3324	33.70
0.3	52108 ± 1463	21.20
0.15	55721 ± 73	15.70
0.07	56820 ± 152	14.10

* Cells were cultured as described in the Methods.

TNF was added at the beginning of the culture and ^{3}H-TdR uptake was determined on day 5 (n=3; in triplicates).

TABLE III-7

^{125}I-CSF-1 binding# on HL-60 cells after treatment with TNF.

	cpm ± SD	
Treatment	Non-specific	Total
None	1109 ± 133	1238 ± 202*
IFN (100U/ml)	1266 ± 198	5575 ± 424*
TNF (2U/ml)	786 ± 280	5579 ± 982@
TNF (2U/ml)	852 ± 264	6594 ± 814*
IFN + TNF	934 ± 332	4599 ± 694*

The iodinated preparation was 35% bioactive compared to normal controls

* Binding, as described in detail in the Methods, was carried out on day 10 of culture

@ Binding carried out on day 5 of culture

TABLE III-8

IFN and TNF do not synergize to upregulate CSF-1 responsiveness of HL-60 cells even if low levels of produced TNF interact with low levels of IFN.

Treatment*			^{3}H-TdR incorporation
IFN (U/ml)	TNF (ng/ml)	CSF-1 (U/ml)	(cpm ± SD) on day 10
--	--	--	141872 ± 5614
100	--	--	109890 ± 4352
10	--	--	130923 ± 6672
1	--	--	132860 ± 5916
100	--	1000	140046 ± 2854
10	--	1000	117130 ± 8131
1	--	1000	131865 ± 6135
--	0.5	--	52014 ± 2460
--	--	1000	143982 ± 3428
--	0.5	1000	44242 ± 707
100	0.5	--	52852 ± 2684
10	0.5	--	54482 ± 1555
1	0.5	--	44104 ± 1472
100	0.5	1000	44770 ± 1458
10	0.5	1000	39412 ± 2904
1	0.5	1000	41310 ± 2926

* These results are representative of one experiment out of a series of three. All tests were carried out in triplicate.

low level of TNF production co-ordinately with the monocytic differentiation of HL-60 cells, TNF is in no way involved in mediating the IFN-induced up-regulation of HL-60 growth response to CSF-1. However, the possibility that TNF produced due to IFN is associated at the membrane or cytoplasm level of the cells cannot be excluded.

Assay of supernatants from IFN-treated HL-60 cells for IL-1 by the very sensitive NOB-1/CTLL co-culture method (Gearing et al., 1987) also showed absence of IL-1 production (Appendix 6). Even though the slight IL-1 stimulation increases in HL-60 growth counter-indicates differentiation to a CSF-1 receptor-bearing phenotype, we non-the-less asked whether IL-1 and IFN might co-operatively stimulate response to CSF-1. Addition of IL-1 to IFN-treated cells at several levels of IFN with and without CSF-1 (a parallel to the TNF experiment in Table III-8) showed that IFN did not alter control responses (Appendix 7). We therefore concluded that IL-1, like TNF, does not mediate the IFN-induced up-regulation of CSF-1 growth response in HL-60 cells.

Time requirement for IFN and CSF-1 action

Up-regulation of mRNA levels encoding the CSF-1 receptor (Sariban et al., 1985 & 1987) and of response to CSF-1 begins only after 7-8 days of incubation with IFN and continues to increase through day 10. Unknown is whether IFN is required for the entire 7-day lag period or only later for maintenance of receptor expression, and whether CSF-1 is necessary for the increase in receptor expression past day 8. The effect of IFN in cultures for 1, 2, 3, 4 and 5 days on the ability of the cells to respond to CSF-1 by day 10 was tested. We found that the minimum induction period is two days (Table III-9). These results show that IFN is required for only 48 hr to initiate

TABLE III-9

Time requirement for IFN's action on the up-regulation of CSF-1 response.

Days with IFN	Addition of CSF-1	^{3}H-TdR incorporation (cpm ± SD) on day 8*,#	Percent increase
0	--	75720 ± 2128	
0	+	74108 ± 2663	-2.1
1	--	68893 ± 376	
1	+	64588 ± 3368	-6.2
2	--	64148 ± 948	
2	+	84742 ± 1476	32.0
3	--	65322 ± 1212	
3	+	86218 ± 1416	32.0
4	--	61318 ± 1106	
4	+	83559 ± 318	36.0
5	--	68411 ± 309	
5	+	81522 ± 1740	19.0

* These results represent one experiment out of a series of three. Cpm ± SD are from triplicate wells.

The cells were cultured with IFN for the indicated number of days and then CSF-1 at 1000U/ml was added. ^{3}H-TdR incorporation was monitored every day thereafter. The results presented in this table correspond to:

x days with IFN + z days with CSF-1 = 8

differentiation that begins to manifest itself as CSF-1 responsiveness only 6 days later.

In order to determine whether CSF-1 is needed for maintenance of functional receptor after an initial two or five day induction period with IFN, cells were washed at day 2 or 5 then subcultured with and without CSF-1 and IFN. The results indicate that neither IFN nor CSF-1 is necessary for maintenance of functional receptors (binding of CSF-1 at day 10) after induction has taken place (Table III-10).

Binding experiments indicate that untreated cells express a low level of high affinity-binding sites. This is in accord with the observations of Weber et al. (1988), that c-*fms* transcripts are present at very low levels in HL-60 cells. Autoradiographic data on the fraction of cells able to bind ^{125}I-CSF-1 (see Fig. III-1) indicates that the difference in overall receptor levels between IFN-treated and untreated HL-60 cells is due to the number of cells expressing high-affinity binding (about 9 % for untreated and 33 % for treated).

TABLE III-10

Neither IFN nor CSF-1 is necessary for the maintenance of CSF-1 receptor levels.

Induction+ by:	Addition of CSF-1 (on day 5)	Receptors/cell* (± SD)
Nothing	--	328 ± 36
Nothing	+	352 ± 48
CSF-1	--	333 ± 56
CSF-1	+	301 ± 21
IFN+CSF-1	--	1205 ± 110
IFN+CSF-1	+	1182 ± 202
IFN	--	1696 ± 301
IFN	+	1306 ± 315

+ Cells were cultured for 5 days with the indicated factors (CSF-1 at 1000U/ml, IFN at 100U/ml), then washed and subcultured with and without CSF-1 as indicated.

* Determined on day 10 as detailed in the Methods.

[D]. DISCUSSION

In this study we examined the up-regulation of the human CSF-1 receptor during the differentiation of HL-60 cells after induction with IFN. Because little is known about the up-regulation of the CSF-1 receptor and its role on leukemic cells, we used the inducible HL-60 cell line as a model to study this event. Our rationale was based on three known facts: a) The ability of IFN to induce monocytic differentiation in this leukemic cell line model (Ameglio et al., 1983; Rosa & Fellous, 1984; Becker, 1984; Trinchieri & Peroussia, 1985), b) The induction of c-*fms* and TNF mRNA on HL-60 after IFN treatment (Sariban et al., 1985 & 1987) and c) That c-*fms* expression has been assigned as a molecular marker in human acute myeloid leukemias (AML; Dubreuil et al., 1988).

The effect of IFN on morphology, phagocytic activity and surface marker expression on HL-60 cells has been previously studied by other groups (reviewed by Collins, 1987). Here, we present a quantitative analysis of these effects. Seven days after IFN treatment almost 50% of the cells were able to injest fluorescent beads and exhibit monocytic characteristics. The treatment also increased class II expression and more than 30% of the cells were induced to express CD 14 (Table III-1), a myelomonocytic differentiation antigen expressed by monocytes, macrophages and activated granulocytes (Goyert et al., 1988). This induction of a lineage-specific surface antigen may have a more complex function than initially anticipated since the gene encoding the CD 14 molecule is located on the "hot region" of chromosome 5 known to encode several myeloid-specific growth factors or their receptors (Simmons et al., 1989). Tac antigen expression was also observed and correlates with findings previously described (Herrman et al.,

1985; Vassiliadis et al., 1989). These are all independent characteristics of monocytic differentiation. Thus, it appears that IFN acts in a rather pleiotropic manner to initiate a process of differentiation in a cell line blocked at a highly proliferative stage of differentiation (Collins et al., 1978; Breitman et al., 1980).

For this study, the marker of monocytic differentiation was the CSF-1 receptor which is being induced by IFN. Although accumulation of CSF-1 receptor mRNA (c-*fms*) has been reported by other groups (Sariban et al., 1985 & 1987), the presence of a functional CSF-1 receptor has not yet been described. Therefore, IFN-treated cells were tested for their ability to bind and respond to CSF-1. ^{125}I-CSF-1 binding experiments showed that the number of functional receptors per cell increased considerably after treatment reaching 2,000 receptors/cell (Table III-3; or 6000 receptors/cell considering that only 33% of the population acquires the receptor) 9 days post-induction. Responsiveness to this lineage-specific factor appeared 8 days after addition and reached higher levels by day 10, as tested by ^{3}H-TdR incorporation (Table III-2). Autoradiographic analysis showed that 37% of the cells could incorporate ^{3}H-TdR in response to CSF-1 after the treatment (Table III-5), and that 33% of the induced cells could bind iodinated CSF-1 (Fig. III-1). Similar results were obtained when the clonogenic capability of HL-60 cells was tested (Table III-2).

Uninduced HL-60 cells appeared to be insensitive to CSF-1 action (Table III-2). It has been reported, however, that all primary "M" subclasses of AML express c-*fms* and respond to CSF-1 in various degrees (Dubreuil et al., 1988). Although our cells did not respond to CSF-1, we were able to detect a low amount of binding (about 9 % of the cells) that confirms the above observations. The fact that no response is obtained may be attributed

to the bulk (91 %) cell population that does not bind CSF-1 and, at the same time, is able to proliferate in culture thus masking a small effect.

It has been established so far that the cells become responsive to CSF-1 after IFN treatment and a lag period of several days. It is not clear why the up-regulation of the CSF-1 receptor appears so late. The Kufe group (Sariban et al., 1987) proposed that the expression of c-*fos*, c-*fms*, and c-*sis* in IFN-induced HL-60 differentiation, is probably related to the maintenance, progression and/or function of the differentiated phenotype rather than induction along the monocytic pathway. It is reasonable to speculate that a number of events has to be completed within a certain period of time before CSF-1 receptor expression. In order to determine the real inductive capacity of IFN and to calculate the lag period that precedes CSF-1 responsiveness, we performed kinetic experiments by removing IFN from the cultures. We found that the IFN requirement for up-regulating the CSF-1 response was only 48 hr (Table III-9). Induction of CSF-1 responsiveness is mediated by IFN only as other IFN-produced and/or IFN-synergistic "partner-cytokines" like TNF and IL-1 (Newton, 1985; Trinchieri et al., 1986; Dower & Urdal, 1987; Tsai & Gaffney, 1987; Fibbe et al., 1989) were shown not to be involved in this process (Tables III-5, -7, -8).

It is interesting that CSF-1 itself is not required after the IFN induction for up-regulating its own response. Results obtained from a series of experiments indicated that the HL-60 cells had equal capabilities of binding iodinated CSF-1 in the presence and/or absence of this factor (Table III-10).

In summary, our data indicate that IFN triggers a series of events during the first two days of culture that lead to a later up-regulation of functional CSF-1 receptors. The induced CSF-1 receptor is the mediator for signal

transduction upon addition of CSF-1 that results in a higher cell growth rate. Because removal of IFN from the cultures did not inhibit the program of differentiation already started, the high potential of IFN as a differentiation agent in human leukemia is indicated. However, the IFN's effect is not complete. Unknown is whether complete differentiation to a nongrowing state can be achieved if these intermediates state cells are exposed to other agents. One logical candidate is CSF-1, reputed to limit the growth of myeloid leukemia cells. This study is the subject of a following communication (see chapter V).

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CHAPTER IV

IMMUNE INTERFERON INDUCES EARLY AND LATE EVENTS IN DIFFERENTIATION OF HL-60 THAT ARE PART OF THE SAME MATURATION PROGRAM

[A]. INTRODUCTION

Thirty seven percent of the bulk HL-60 population is induced by immune interferon (IFN) to express functional colony-stimulating factor-1 (CSF-1) receptors. A two-day treatment with IFN sets in motion a differentiation program which leads to an early and transient increase of human leukocyte antigen-DR (HLA-DR) and a delayed appearance of CSF-1 receptors. In this study we have investigated the inverse relationship between class II and CSF-1 receptor expression and found that IFN acts only on an IFN-sensitive HL-60 cell population that is class II negative. This population becomes susceptible to induction and express the early HLA-DR surface antigen. The same cells become programed to acquire CSF-1 receptors at a later time, an effect that cannot be substituted by another agent (A23187) known to have the IFN's effect on DR expression. Induced class II expression by itself is not sufficient to predict a future CSF-1 receptor appearance suggesting that HLA-DR induction by IFN is an accompanying rather than a causative event, although inhibition of class II completely ablates the IFN-up-regulation of CSF-1 receptors. In contrast, constitutive class II positive cells do not respond to CSF-1 nor they can be induced to do so.

IFN was originally defined as part of non-specific immunity against

viruses, some micro-organisms and tumors. However, it has been found to function as a pleiotropic agent whose roles include the control of cell multiplication (Trinchieri & Perussia, 1985; Inglot, 1983). In this latter role, its effects *in vitro* on the induction of differentiation of immature or leukemic cells to a nongrowing monocytic-like state as assessed by morphology, function and antigen expression has been well studied (Ameglio et al., 1983; Ball et al., 1984a & b; Becker, 1984; Rosa & Fellous, 1984; Sutherland et al., 1985; Collins, 1987). This differentiation-inducing property has been studied mostly by means of cell line models. The human promyelocytic leukemia cell line HL-60 (Collins et al., 1977) can be programed to differentiate towards the monocytic pathway when induced by IFN (reviewed by Collins, 1987). The process of IFN-induced differentiation involves expression of Ca^{2+} / calmodulin-dependent HLA-DR increase in cell surface HLA-DR (Ina et al., 1987) and a delayed expression of mRNA for the product of the proto-oncogene c-*fms* (Sariban et al., 1985 & 1987) which is identical to CSF-1 receptor (Sherr et al., 1985).

Little is known about the regulation of c-*fms* during human normal differentiation. This gene, however, is always expressed in almost all myeloid leukemias (Dubreuil et al., 1988). HL-60 does not express c-*fms* mRNA unless induced to differentiate with agents such as TPA (Sariban et al., 1985). Increase in message level seems to occur at the post-transcriptional level (Weber et al., 1989). When the cells are treated with IFN, the same up-regulation of c-*fms* occurs but at a later time (Sariban et al., 1987). We have shown that c-*fms* is functionally expressed on the surface of HL-60 cells starting 8 days after IFN treatment as the CSF-1 receptor and that IFN is needed for the first 48 hr only (previous chapter). Function has been

demonstrated by direct binding studies with ^{125}I-CSF-1 and by the ability of IFN-treated cells to be mitogenically stimulated by CSF-1 (Kawasaki et al., 1985). The delayed receptor up-regulation by IFN appears to be direct and not mediated by products secreted by IFN-induced cells since repeated washing after a 48 hour induction period by IFN fails to alter receptor up-regulation later (Appendix 5).

The delayed up-regulation of the CSF-1 receptor seems to be a unique response to IFN. IFN is known to transmit differentiation signals very rapidly as is the case with HLA-DR or class I expression (chapters III and II). There is, however, literature that indicates heterogeneity in mechanistic paths involving IFN action. For example, theophylline (TPH), a drug which blocks the Ca^{2+} / calmodulin second messager pathway, blocks IFN-induced class II but not class I expression (Ina et al., 1987 & Appendix 4). Whether the early, direct effects of IFN are mechanistically related to delayed effects on the expression of the CSF-1 receptors, or even occur on the same or different cells, is not known.

We here therefore examine both early and delayed effects induced by IFN on HL-60 cells and ask whether the early events predict the later expression of the CSF-1 receptor and, if so, whether class II (HLA-DR) expression might be sufficient for the later up-regulation of c-*fms*. FACS sorting and analysis shows that induced high DR expression correlates with CSF-1 receptor up-regulation 6 days later. However, early induction of HLA-DR by another agent (the calcium ionophore A23187; Ina et al., 1987) does not cause the expected up-regulation of response to CSF-1 suggesting that class II expression by itself is not a causative event but accompanies other events induced by IFN that are important for the CSF-1 receptor expression.

[B]. MATERIALS AND METHODS

Cells and culture conditions: 1) HL-60 cells (Collins et al., 1977) were obtained from the American Type Culture Collection (ATCC, Rockville, MD), grown in suspension culture in Iscove's modified Dubelcco's medium (IMDM; Gibco Laboratories, Grand Island, NY) containing 2.5% FCS (Flow) at 37° C, in a humidified atmosphere containing 5% CO_2 in air.

2) HL-60 cells, constitutively expressing c-*fms*, were provided by Dr C. Sherr and were obtained by retroviral infection using a c-*fms* neoresistance-containing vector packaged in PA317 cells. After selection in G418, the cells were sorted twice with an antibody to CSF-1 receptor to generate a population of cells positive for the human receptor, T99:SS1-S2. These cells were grown like the mother HL-60 line.

Induction protocols: One hundred thousand HL-60 cells were cultured with 100 U of IFN /ml for five days and then assayed for proliferation, expression of cells surface markers and binding of radiolabeled CSF-1. Other induction protocols were also used for the present study. In particular, induction was carried out with the same amount of IFN but for only two days. This type of induction was used mainly for the sorting experiments.

Interferon: A preparation of human, recombinant IFN was a gift from Drs E. Falcoff and J. Wiezerbin (Institute Pierre et Marie Curie, Paris, France) and used at 100 U/ml.

CSF-1: Recombinant human CSF-1 was obtained from Dr P. Ralph, Cetus Corp., Emeryville, Ca., and used as an unpurified supernatant from CHO cells plasmid-transfected with the truncated form cDNA of Kawasaki et al. (1985). This factor was used at a concentration of 1300 U/ml.

Theophylline: Stock solutions of TPH (Sigma, St. Louis, Mo) were prepared in phoshate buffered saline (PBS) routinely at a final concentration of 0.18 mg/ml, a concentration found to exert maximal effects and minimal toxicity.

Calcium ionophore A23187: A23187 was obtained from Sigma (St. Louis, Mo) and used at a final concentration of 160 nM which was not toxic for the cells as determined by serial dilution and subsequent ^{3}H-TdR incorporation assays.

^{125}I-CSF-1 binding assay: Labeling of CSF-1 was performed by using a modified chloramine T method to achieve a specific activity of about 10^{18} cpm/mol of protein (Guilbert & Stanley, 1986). The iodinated preparation was routinely tested for bioactivity as previously described on growth of factor dependent, murine macrophage cell lines (Branch & Guilbert, 1989).

One hundred thousand cells were cultured in CSF-1-free medium one day before the binding experiments in order to increase the CSF-1 receptor density then washed twice with cold PBS in preparation for the binding reaction. After a 20-minute preincubation time on ice, binding was carried out in 15-ml polypropylene tubes (Corning) at a final volume of 200 to 250 microliters at 4° C in IMDM minus bicarbonate tittered to pH 7.35 with NaOH (ImB). Specific binding was determined as the amount of ^{125}I-CSF-1 binding that could be blocked by a one hour preincubation with 2 nM of unlabeled CSF-1 (Stanley & Guilbert, 1980; Guilbert & Stanley, 1986). The binding reaction was stopped and unbound label separated from bound by centrifuging the cell suspension through an ice-cold FCS layer. After the cells were washed once more in cold PBS, the ^{125}I content of the pellet was determined in a LKB-Wallac CliniGamma 1272 rack counter (Finland).

Because CSF-1 forms an unstable complex at 4° C when it binds to its receptor (there is no K_{off}), Scatchard analysis cannot be performed to determine the number of binding sites/cell. Instead, this number can be calculated by the formula:

$$\text{Receptors/cell} = N \frac{\text{Bound (cpm)}}{\text{S.A x Number of cells employed}}$$

where $N = 6.02 \times 10^{23}$ and S.A = specific activity.

All binding experiments shown here were carried out with preparations having specific activity of about 1×10^{18} cpm/mol. Saturation of all available sites was confirmed by demonstrating that the amount of excess label added in various experiments corresponds to plateau binding levels.

Tritiated Thymidine (^{3}H-TdR) Uptake: Cells were cultured in triplicate wells of flat-bottomed 96 well microculture plates (Linbro, Flow Laboratories, Hamden, CT). Five thousand cells in a final volume of 200 ul were incubated at 37° C in 5% CO_2 in air for up to 7 days. Cultures were pulsed for 4 hr with one uCi/well of ^{3}H-TdR (New England Nuclear, Boston) prior to harvesting and counted on a Packard Minaxi B 4000 liquid scintillation counter (Packard Instruments, Downers Grove, IL). Thymidine uptake is expressed as mean counts per minute (cpm) $\pm$ standard deviation (SD).

Marker Analysis and Sorting: The indirect immunofluorescent analysis of cell surface antigen expression were performed as following: Cells were incubated for one hour at 4° C in the presence of the appropriate antibody (1/500 final dilution of 7H3 for class II, a murine IgG2a; 83H1 for

class I, a mouse IgG1; MacLean et al., 1982; Mannoni et al., 1982) then washed three times with cold PBS and incubated for an hour at the same conditions in the presence of FITC-conjugated goat anti-mouse IgM, IgG and IgE antibody (Cappel Lab., Cochranville, PA). After washing three times, the cells were fixed in 2% formaldehyde and fluorescence intensity of individual cells quantified by flow cytometry using an EPICS V fluocytometer having a 5-watt argon laser (Coulter Electronics, Hialeah FL). Ig subtype matched antibody controls with inappropriate specificity were the T cell specific antibodies: B9.11, a murine IgG1 (CD8) and 88H1 a murine IgG2a (CD7). Both antibody preparations were gifts from Dr. P. Mannoni, France.

For sorting, the cells were stained under sterile conditions with the monoclonal antibody 7H3, where indicated, and kept on ice at a higher FCS concentration (15 %) in order to improve cell viability. The gates for sorting were set according to the histogram analysis shown in Figures 2 and 3. Sorted cells were immediately placed in culture for two to three days in order to recover from the sorting procedure, then used as described in the Results.

[C]. RESULTS

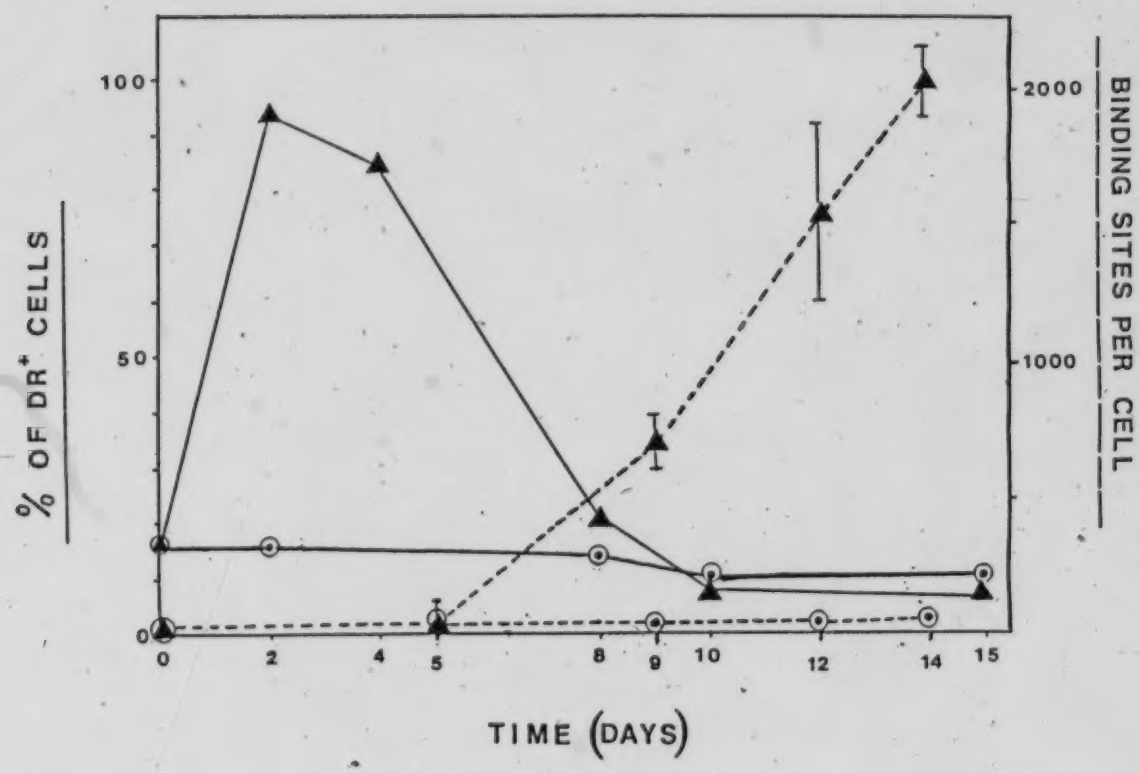
Transient expression of HLA class II and response to CSF-1

Preliminary data from the literature (Rosa & Fellous, 1984) and from our laboratory show that HL-60 cells cultured with IFN express cell surface HLA-DR within 48 hr and functional cell surface CSF-1 receptor only after 8 days (chapter III). Since detailed kinetics of such expression have not been reported previously, we directly compared the time course of cell-surface DR and high-affinity CSF-1 binding-site expression using the same IFN induction protocol. The results (Fig. IV-1) show that expression of HLA-DR is transient, peaking at 48 hr, and is inversely related to the expression of CSF-1 receptors. The order of IFN-induced expression of the two cell-surface molecules (DR first, then the CSF-1 receptor) and their inverse relationship after 48 hr suggested that prior expression of HLA-DR was either closely linked to or required for later expression of surface CSF-1 receptors.

The relationship between peak HLA-DR expression 48 hr after initiation of IFN treatment and the delayed up-regulation of functional CSF-1 receptors was examined in two ways. First, IFN-treated cells were separated by FACS into populations with low and high levels of DR, and functional CSF-1 receptors in these populations were assessed 8 days later. Second, complement-mediated killing was employed at 48 hr, to remove cells expressing high levels of DR after IFN treatment, and 8 days later functional CSF-1 receptors on surviving cells and their descendants were assesssed.

Figure IV-1

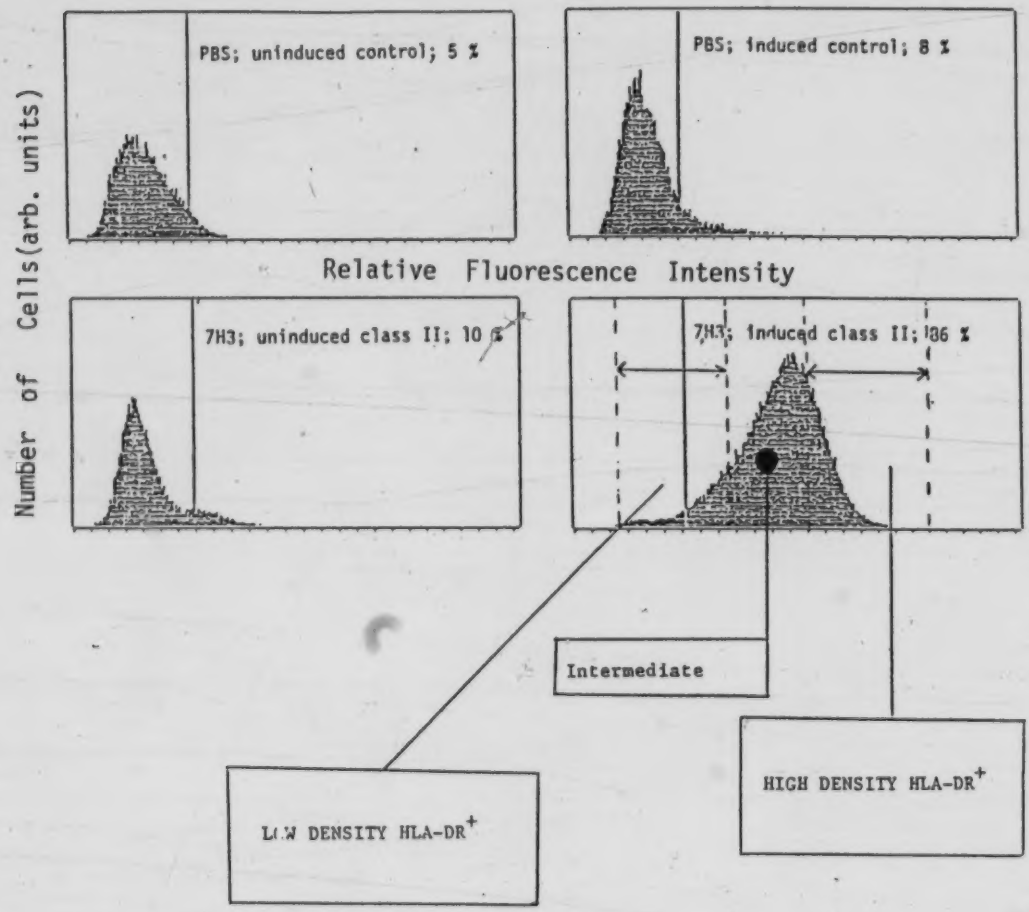
Inverse relationship between class II modulation and CSF-1 binding after IFN treatment. The continuous line represents the kinetics of class II expression and the broken line the kinetics of binding. Triangles indicate treated cells and circles indicate untreated control cells.



In the first approach, HL-60 cells after 48 hr of incubation with 100 U of IFN/ml were sorted by FACS. Indirect immunofluorescence with the anti-DR antibody 7H3 was used to sort them into three populations according to their level of HLA-DR expression (low, intermediate, and high density; see Fig. IV-2). Cells of intermediate density were discarded, and approximately equal sized low and high density populations were put in culture for 3 more days with and without IFN. Once the induction period was completed, they were subcultured again into groups with and without CSF-1. Functional receptor was assessed 5 days later (10 days after initial exposure to IFN) by the relative uptake of ^{3}H-TdR with and without CSF-1 (Table IV-1). The results, expressed as percent increase in ^{3}H-TdR in response to CSF-1 within a treatment group, show that IFN-treated cells that bear low (or zero) HLA-DR levels at 48 hr remained unresponsive to CSF-1 8 days later, whereas cells expressing high levels were responsive. The data in Table IV-1 demonstrates again that IFN is needed for only 48 hr for the later up-regulation of CSF-1 responsiveness. That expression of HLA-DR on a cell (or a descendant) correlates with its later expression of functional CSF-1 receptor was confirmed by a second experiment involving complement-mediated killing of IFN-treated cells expressing HLA-DR after 48 hr of culture (Table IV-2). Although complement alone reduced CSF-1 responsiveness by half, complement plus anti-HLA-DR completely eliminated cells that became responsive to CSF-1 later in culture. The data in Tables IV-1 and IV-2 show that IFN-treated HL-60 cells expressing low, or null, cell surface levels of class II after 48 hr of culture were unable to manifest the delayed up-regulation of functional CSF-1 receptor 3 to 8 days later. These results strongly argue that IFN-induced expression of functional CSF-1 receptor either requires prior expression of HLA-DR or that DR

Figure IV-2

Sorting HL-60 populations for positive and negative class II cells after exposure to 100 U/ml of IFN for 2 days.


PBS; uninduced control; 5 %
PBS; induced control; 8 %
Number of Cells(arb. units)
Relative Fluorescence Intensity
7H3; uninduced class II; 10 %
7H3; induced class II; 36 %
Intermediate
HIGH DENSITY HLA-DR+
LOW DENSITY HLA-DR+

The cells were tagged with the anti-class II monoclonal antibody 7H3 (MacLean et al., 1982) as described in the methods. PBS substituted for 7H3 in controls as the primary antibody.

TABLE IV-1

Responsiveness of HLA-DR-positive and -negative sorted HL-60 cells towards CSF-1 after induction with IFN*.

	Culture components		^{3}H-TdR uptake	% increase
	IFN	CSF-1	(cpm ± SD)	in response to CSF-1
DR low	--	--	2758 ± 80	
	--	+	2794 ± 166	1.3
	+	--	1348 ± 42	
	+	+	1387 ± 177	3.0
DR high	--	--	1306 ± 98	
	--	+	1988 ± 226	53.0
	+	--	1002 ± 44	
	+	+	1699 ± 105	69.0

* On day 0, the cells were cultured with 100 U/ml of IFN. On day 2, the cells were sorted with the monoclonal antibody 7H3 into populations expressing high and low densities of HLA-DR (positive for DR: 64% of treated cells versus 14% of control; see Methods for details on FACS analysis, and Fig. 2 for criteria of sorting). After sorting, the cells were immediately divided into 8 groups for various treatments with and without IFN. On day 5, CSF-1 was added (1000 U/ml) to half of the cultures; and on day 10, responsiveness to this factor was determined by ^{3}H-TdR incorporation.

TABLE IV-2

CSF-1 responsiveness of cells treated with anti-class II monoclonal antibody plus complement*.

A Cells treated with anti-class II plus complement.

Culture Components		^{3}H-TdR incorporation	% increase
IFN	CSF-1	(cpm ± SD; day 8)	to CSF-1
--	--	42953 ± 951	
--	+	41513 ± 581	
+	--	34694 ± 823	
+	+	36528 ± 1438	5.0
B	Cells treated only with Complement:		
--	--	45432 ± 1126	
--	+	46814 ± 2412	
+	--	40024 ± 982	
+	+	46356 ± 744	16.0
C	Control HL-60 cells:		
--	--	72632 ± 562	
--	+	71131 ± 215	
+	--	67202 ± 1010	
+	+	99855 ± 1720	33.0

* The cells were treated and cultured as described in Results.

expression at 48 hr culminates in expression of CSF-1 receptor 6 days later. The following experiments aim at demonstrating these two possibilities.

Inhibition of early class II expression inhibits later CSF-1 responsiveness

We first asked whether inhibition of IFN-induced HLA-DR expression could also inhibit expression of CSF-1 receptor. It is known that signals transduced via the Ca^{2+} / calmodulin second messenger pathway appear to be necessary for the induction of class II expression on HL-60 cells by IFN (Ina et al., 1987). The drug TPH that blocks this pathway by inhibiting the enzymes interacting with calmodulin also effectively blocks the IFN's action in inducing class II. Therefore, in order to determine whether the Ca^{2+}/calmodulin second messenger pathway is important to both DR expression and CSF-1 responsiveness we used the calmodulin inhibitor TPH to block IFN-induced expression of HLA-DR as described by Ina et al., (1987). We first confirmed that TPH blocked the IFN-induced expression of HLA-DR after 48 hr of treatment (52% with IFN versus 22% with the combination of IFN and TPH), but did not affect expression of other markers (Table IV-3; Appendix 2). We then maintained the same cells in culture for the full 5 day induction period with IFN, then transferred to CSF-1 containing medium to quantitate the response to CSF-1 on day 10 . The results show that TPH slowed the growth of HL-60 cells with or without IFN, and completely ablated the response of IFN-treated cells to CSF-1 (Table IV-4). In addition, cells treated with TPH from days 0 to 5 (when TPH blocks IFN's effect on class II) do not upregulate ^{125}I-CSF-1 binding sites in

TABLE IV-3

The effect of theophylline (TPH) on IFN-induced expression of cell surface antigen expression on HL-60 cells after 48 hr of treatment.

	Percent of positive cells*		
Culture components	PBS	Class II	CD 14@
Nothing	10	16	8
IFN	5	52	31
TPH	3	22	28
TPH + IFN	2	22	31

* Determined by indirect immunofluorescent analysis on FACS as described in the Methods.

@ The monoclonal antibodies used were: 7H3 (IgG 2a) for class II, 82H3 (IgG 1) for CD 14, and PBS as negative control. T cell specific antibodies of same isotype were also used as controls (described in Methods).

TABLE IV-4

Effect of 0.18 mg/ml theophylline on IFN-induced up-regulation of functional CSF-1 receptors on IFN-treated HL-60 cells.

Culture components			^{3}H TdR incorporation	CSF-1 Receptors/cell(±SD)#
CSF-1	IFN@	TPH	(cpm±SD)	
--	--	--	52542 ± 381	437 ± 54
+	--	--	54016 ± 476	250 ± 22
--	+	--	45323 ± 381	1618 ± 77
+	+	--	59586 ± 222	1347 ± 68
--	--	+*	30556 ± 986	132 ± 15
+	--	+*	32034 ± 497	113 ± 20
--	+	+*	24854 ± 468	187 ± 65
+	+	+*	22756 ± 905	276 ± 98
+	+	+&	58541 ± 123	1224 ± 106
--	--	+&	49877 ± 305	205 ± 55
--	+	+$	26556 ± 408	1176 ± 144
--	--	+$	33476 ± 654	167 ± 97

@ Where indicated IFN was present throughout the culture at 100 U/ml.

* TPH was added from the beginning of the culture

& TPH was added one day before the test

$ TPH was added after the 5-day induction period

Numbers of CSF-1 receptors/cell determined from specific binding as detailed in the Methods.

response to IFN as the control cells do (Table IV-4). TPH treatment does not affect the mitogenic action of CSF-1 and its binding if TPH is added after class II has already been expressed (after day 2; Table IV-4).

In order to exclude the possibility that TPH inhibits cell-surface expression of intracellular CSF-1 receptor, we TPH-treated the human choriocarcinoma line JAR (Rettenmier et al., 1986), which both produces and binds CSF-1 and therefore turns its receptor complement over continuously, and determined binding of CSF-1. We found that TPH did not inhibit the binding of CSF-1 (824 ± 108 cps with TPH versus 801 ± 79 in the control) suggesting that TPH does not interfere with membrane expression. The results in Tables IV-3 and 4 point to the importance of the Ca^{2+} second messenger pathway both in regulating class II expression after 48 hr of IFN treatment and in later up-regulation of functional CSF-1 receptors and indicate similar mechanistic pathways for both processes.

Class II expression is not sufficient for the up-regulation of CSF-1 receptors after IFN treatment of HL-60 cells

We next asked whether increase in intracellular Ca^{2+} concentration would up-regulate both HLA-DR and CSF-1 receptors. A23187 has been reported to increase class II expression on these cells by increasing the intracellular levels of calcium (Ina et al., 1987). As reported , we observed an increase in class II on days one and two (from 6% control value to 59% with A23187) whereas co-culture of IFN and A23187 did not change the percentage for the surface antigen, class II (approximately 60%). We then

asked whether A23187-treated HL-60 cells would up-regulate responsiveness to CSF-1 with the same kinetics as IFN-treated cells. For this, we treated the HL-60 cells with A23187 for two days in the absence of IFN and tested CSF-1 responsiveness on day 10 by ^{3}H-TdR incorporation. Our results (Table IV-5) showed that regardless of the induced class II expression on day 2 (Table IV-3), the cells did not become CSF-1 responsive 6 to 9 days later as did the control IFN-treated group. In order to exclude the possibility that A23187 blocked the response to CSF-1, we treated a mouse bone-marrow derived macrophage cell line that requires CSF-1 for growth (S1 cells; Branch et al., 1989) with A23187 and tested their ability to respond to CSF-1. We found that this agent did not at all impair the CSF-1-stimulated growth of these cells (14252 $\pm$ 1421 cpm with CSF-1 versus 15039 $\pm$ 560 cpm with CSF-1 + A23187 on a two day ^{3}H-TdR assay). In addition, A23187 was not able to block CSF-1 responsiveness of the HL-60 variant T99:SS1-S2 that constitutively expresses the CSF-1 receptor (33188 $\pm$ 1576 cpm with CSF-1, 35996 $\pm$ 1712 with CSF-1 + A23187, and 18324 $\pm$ 524 the control; on a 3-day proliferation assay). These data indicate that A23187 does not non-specifically inhibit CSF-1 responsiveness.

Taken together, the above observations indicate that class II expression alone is not sufficient for induction of functional CSF-1 receptors and subsequent response to CSF-1 and that other mechanisms than, or in addition to, class II expression are involved. In order to further investigate this point, we sorted approximately 10% of untreated HL-60 cells that constitutively expressed class II into extreme groups expressing very little and very high levels of DR (see Fig. IV-3 for sorting criteria) and asked whether either of these two cell sets could respond to CSF-1 without IFN induction.

TABLE IV-5

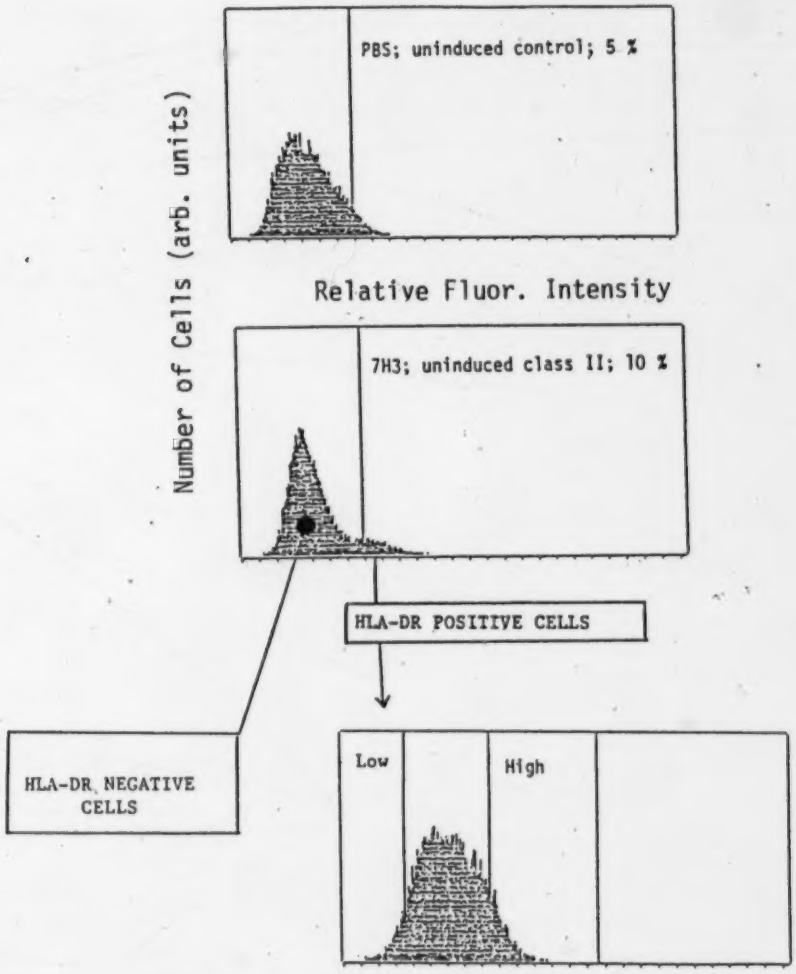
Effect of calcium ionophore A23187 on up-regulation of responsiveness to CSF-1.*

Culture components			^{3}H-TdR incorporation
CSF-1	A23187	IFN	(cpm ± SD) on day 10
--	--	--	107694 ± 564
+	--	--	106434 ± 821
--	+	--	81086 ± 4516
+	+	--	79687 ± 2284
--	--	+	64118 ± 2146
+	--	+	103176 ± 1886

* The cells were treated with 100 U/ml of IFN or 160 nM of A23187 for two days. CSF-1 was added at 1000 U/ml after the induction and stayed in culture up to day 10.

Figure IV-3

Sorting of HL-60 cells that constitutively express HLA-DR into low- and high-density groups.



The data in Table IV-6 show that neither extreme population responded to CSF-1 nor did the intermediate population or combination of the groups (see Table IV-7). These results show that 1) CSF-1 responsiveness requires IFN induction and 2) constitutive class II expression does not predict a future CSF-1 response.

In order to determine whether these separated populations of HL-60 cells could non-the-less be induced by IFN to up-regulate CSF-1 responsiveness, we cultured them after sorting (see Fig. IV-3 for criteria) for 5 days with and without IFN, then tested for response to CSF-1 by subculturing for 5 more days with and without CSF-1 (Table IV-7). Surprisingly neither of the extreme HLA-DR populations was able to be induced by the IFN treatment and to up-regulate a CSF-1 response. However, sorting of the DR-negative cell population and subsequent induction with IFN and addition of CSF-1 later revealed that these cells were permissive to IFN's action since they demonstrated a reduced growth rate in response to IFN (about 30 %), expressed class II on day two (approx. 54%) and became CSF-1 responsive (about 60 % above the IFN control) as anticipated at a later time (Table IV-7). These results show that IFN acts only on the class II-negative HL-60 cells that are being programed to differentiate towards the monocytic pathway.

TABLE IV-6

CSF-1 response of untreated HL-60 cells, sorted into high and low density HLA-DR positive groups.*

Population	Factor	^{3}H-TdR incorporation (cpm ± SD) on day 5
LOW (left)+	none	18550 ± 985
	CSF-1	17405 ± 1605
HIGH (right)+	none	15290 ± 1810
	CSF-1	13101 ± 855

+ Refers to groups defined in Fig. IV-3.

* The sorting and the reagents used for the analysis are described in detail in the Methods.

TABLE IV-7

CSF-1 response of untreated HL-60 cells, sorted into groups expressing no HLA-DR and very low as well as very high DR-density subgroups.*

* After sorting the cells were treated for 5 days with 100 U of IFN/ml (where indicated) and subsequently subcultured with or without CSF-1 for another 5-day period. This table shows two different experiments; one performed after sorting the DR-positive cells and the other after sorting the DR-negative population (refer to Fig. IV-3 for sorting criteria).

Population	IFN Induction after sorting	CSF-1	^{3}H-TdR incorporation (cpm ± SD) on day 10
LOW (left; DR$^+$)	no	--	26118 ± 288
		+	22763 ± 238
	yes	--	27287 ± 589
		+	22420 ± 393
HIGH (right; DR$^+$)	no	--	24225 ± 725
		+	24326 ± 118
	yes	--	25168 ± 288
		+	24490 ± 154
HLA-DR$^-$	no	--	54326 ± 1582
		+	56887 ± 2107
	yes	--	39101 ± 3453
		+	62244 ± 1866

[D]. DISCUSSION

IFN induces the human promyelocytic leukemia cell line HL-60 to express a number of characteristics typical of monocytes including an early (48 hr) expression of cell-surface HLA-DR, IL-2 receptors, CD 14 and CD 16 expression (Rosa & Fellous, 1984, Vassiliadis et al., 1989, chapter III), as well as a late appearance of monocytoid morphologies and expression of mRNA encoding the CSF-1 receptor (Sariban et al., 1987) and its functional cell surface protein product (previous chapter). We have recently demonstrated that the 'late' IFN-induced events require only 48 hr of IFN treatment for maximal expression (chapter III). This paper examines the relationship between the early and late IFN-induced events in HL-60.

Using cell-surface expression of HLA-DR protein as indicator of early events, we show that it is transient, peaking at 48 hr and inversely related thereafter to a later event, i.e., appearance of functional cell surface CSF-1 receptors (Fig. IV-1). The inverse expression of the representative early and late events suggested either a causative (CSF-1 receptor expression requires prior HLA-DR expression) or a parallel (the underlying mechanisms occur during the 48 hr of IFN treatment and are required for later up-regulation of CSF-1 receptor plus the early and transient expression of DR) relationship. The relationship between the early and late events was firmly established by showing that only the HLA-DR-positive population after 48 hr of IFN treatment goes on to yield functional CSF-1 receptors in culture (Table IV-1), that DR negative cells at 48 hr do not (Tables IV-1 and IV-2) and by

demonstrating that blocking early DR expression with the drug theophylline also blocks later CSF-1 expression (Tables IV-3 and -4). The absence of a causative relationship between the two events was shown by the inability of the cells to respond to CSF-1 after induction of cell surface DR by the calcium ionophore A23187 (Ina et al., 1987; Table IV-5). A further demonstration that HLA-DR expression *per se* is not sufficient for CSF-1 receptor expression was the inability of the subpopulation of untreated HL-60 cells, that express high levels of DR constitutively, to respond to CSF-1 (Table IV-6). We conclude from these data and from the numerous controls described in the Results that the transient induction of HLA-DR expression by IFN and the short-term triggering of later expression of functional CSF-1 receptors are manifestations of the 48 hr process.

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CHAPTER V

CSF-1 AND IMMUNE INTERFERON SYNERGIZE TO INDUCE DIFFERENTIATION OF LEUKEMIC CELLS BEARING CSF-1 RECEPTORS.

[A]. INTRODUCTION

Human promyelocytic leukemia cells (HL-60) expressing the colony-stimulating factor-1 (CSF-1) receptor either by immune interferon (IFN) induction or genetic manipulation may come under normal control after a long-term treatment with IFN and CSF-1. These differentiated cells resemble their normal counterparts since they exhibit a highly granulated cytoplasm which occupies most of the cell surface area, compared to the retracted nucleus, and do not incorporate ^{3}H-TdR as the untreated cells do. The combination of IFN and CSF-1 seems to be necessary to keep these cells alive in culture. The percentage of the cells that can be driven to proliferative senescence is low (40%) and requires a long treatment period ranging from 20 days to 2 months depending on the cell population and perhaps the density of the cell-surface CSF-1 receptor. We believe that expression of CSF-1 receptors plays an important role in the regulation of normal cell growth.

There are many similarities between the human mononuclear phagocytes occurring in the three body compartments: the marrow, the periphery, and the tissues (van Furth et al., 1979). The most immature cell identified in man is the promonocyte, which is a large cell with a high

nucleus-to-cytoplasm ratio (Ackerman & Douglas, 1984). The direct progeny of monoblasts, promonocytes, incorporate ^{3}H-TdR showing that they are actively dividing cells. In contrast, the most mature of the macrophage precursors, monocytes, have a very low labeling index, indicating that they have lost the ability to divide. Fully functional tissue macrophages, however, such as Kuppfer cells, although reported to undergo limited proliferation in the mouse, do not in man (van Furth et al.,.1979).

The cells of the mononuclear phagocyte system respond to the CSF-1 (Kawasaki et al., 1985) which is a lineage-specific factor (Tushinski et al., 1982; Guilbert, 1985). Although the effect of CSF-1 has been extensively studied on the murine system (Stanley & Guilbert, 1980; Guilbert, 1985; Guilbert & Stanley, 1986), little is known about its action on human cells and its importance -if any- to human acute myeloid leukemia (AML). CSF-1 is able to stimulate low levels of growth only in cells from some AML cases (Pèbusque et al., 1988) although all AML subclasses express c-*fms* (Dubreuil et al., 1988). In contrast, no effect on cell morphology or terminal differentiation has been reported in the above mentioned AML cases. It has also been shown (Miyauchi et al., 1988) that CSF-1 is capable of inducing proliferation and some degree of differentiation by increasing the adherent capacity of some AML blast cells grown in suspension culture. This suggests that CSF-1 selects the cells for terminal divisions. A point particularly mentioned in all studies was the heterogeneity of the cell populations that may not have allowed for the CSF-1 effect to be maximal.

In this study we therefore examine the role of CSF-1 on the differentiation of cells from the promyelocytic leukemia cell line HL-60 (Collins, 1987). HL-60 cells can be induced by IFN towards the monocytic

pathway (Ameglio et al., 1983; Rosa & Fellous, 1984). IFN also triggers a slow and reproducible process of differentiation which involves up-regulation of functional CSF-1 receptors as assessed by response and binding to CSF-1 (Vassiliadis et al., 1989a). However, the isolation of a pure CSF-1 receptor-positive HL-60 population after IFN treatment and subsequent sorting has proven difficult because the CSF-1 receptor is only detected transiently (see Results). In order to circumvent this problem, we employed an HL-60-derived cell line (T99:SS1-S2) that expresses the CSF-1 receptor constitutively (Sherr, personal communication).

After characterization of CSF-1 responsiveness, binding capacity of CSF-1 and cell-surface CSF-1 receptor-density these two models were used to ask whether CSF-1 could act as a differentiation factor in potentiating an irreversible maturation pattern in CSF-1 receptor-bearing cell populations.

We found that IFN was able to induce the cells to a monocytic-like state whereas CSF-1 was unable to cause any degree of morphologic differentiation. However, after prolonged treatment with both IFN and CSF-1, the cells lost their proliferative capacity and at the same time their morphology indicated mature myeloid characteristics, i.e., cells of all myeloid classes. A certain percent of these cells show a very limited growth potential and growth factor requirement.

[B]. MATERIALS AND METHODS

Cells: HL-60 cells, constitutively expressing c-*fms*, were provided by Dr C. Sherr and were obtained by retroviral infection using a c-*fms* neoresistance-containing vector packaged in PA317 cells. After selection in G418, the cells were sorted twice with an antibody to CSF-1 receptor to generate a population of cells positive for the human receptor, T99:SS1-S2, hereafter referred as T99 (Sherr, personal communication). The HL-60 cell line (Collins, 1987) was obtained from ATCC (Rockville, Maryland). Both populations were grown in suspension culture in Iscove's modified Dubelcco's medium (IMDM; Gibco Laboratories, N.Y.) supplemented with 2.5% FCS (Flow) at 37° C, in a humidified atmosphere containing 5% CO_2 in air.

IFN: Human recombinant IFN was a gift from Drs E. Falcoff and J. Wiezerbin (Institute Pierre et Marie Curie, Paris France) and was used at a concentration of 100 units/ml.

CSF-1: Recombinant human CSF-1 was obtained from Dr P. Ralph, Cetus Corp., Emervylle, Ca., and used as an unpurified supernatant from CHO cells plasmid-transfected using the truncated form cDNA of Kawasaki et al., (1985). CSF-1 was tested at various concentrations and the final working concentration was 1,000 units per ml of culture.

Colony formation in methylcellulose: Five thousand T99 cells per dish were plated on methylcellulose (final methocel concentration 0.8% w/w) in 35 mm culture dishes (Falcon). Each dish contained the following ingredients: the cells under test immobilized in the viscous culture medium

(alpha-MEM and methylcellulose), 5% FCS, and where necessary CSF-1. All the components were added to a Falcon plastic test tube in a total volume of 3 mls which was mixed well by vortexing and dispensed in one-ml aliquots into Falcon plastic petri dishes using a syringe with a $18^{1/2}$ G needle. The cultures were incubated at 37° C in 5% CO_2 in air and the colonies (aggregates of more than 48 cells) were scored on day 9 using an Olympus inverted microscope.

Tritiated Thymidine (^{3}H-TdR) Uptake: Cell cultures were carried out in triplicate wells of flat-bottomed microculture plates (Linbro, Flow Labs, Hamden, CT) containing 5 x 10^3 cells per well in a final volume of 0.2 ml and were incubated at 37° C in 5% CO_2 in air for up to 6 days. Cultures were pulsed for 4 hrs with one microCi/well of ^{3}H-TdR (New England Nuclear, Boston) prior to harvesting and counted on a Packard Minaxi B 4000 liquid scintillation counter (Packard Instruments, Downers Grove, IL). ^{3}H-TdR uptake is expressed as mean counts per minute (cpm) $\pm$ standard deviation (SD).

^{125}I-CSF-1 binding: CSF-1 was labeled with ^{125}I using a modified chloramine T method to achieve a specific activity of approximately 10^{18} cpm/mol of protein (Stanley & Guilbert, 1980). The iodinated preparation was tested for bioactivity on CSF-1 responsive cells and used only when its minimum level of activity was at least 20% compared to unlabeled CSF-1 controls. Cells were cultured in CSF-1-free medium one day before the binding experiments in order to upregulate the CSF-1 receptor density and then washed twice with cold phosphate buffered saline (PBS) in preparation for the binding reaction. After a 20-minute preincubation time on ice, binding was carried out in 15-ml polypropylene tubes (Corning) at a final volume of 0.2 ml at 4° C in IMDM without any bicarbonate tittered to pH

7.35 with NaOH (ImB). Specific binding was determined as the amount of ^{125}I-CSF-1 binding that was blocked by a 30-minute preincubation with 2 nM of unlabeled CSF-1 (Stanley & Guilbert, 1980; Guilbert & Stanley, 1986). The binding reaction was stopped and unbound label separated from bound by centrifuging the cell suspension through an ice-cold FCS layer. The ^{125}I content of the pellet was determined in a LKB-Wallac CliniGamma 1272 rack counter (Finland).

Marker analysis: The indirect immunofluorescent tests were performed as follows. T99 cells were first fixed in 2% formaldehyde and then incubated for 30 minutes at 4° C in the presence of the appropriate antibody (12-2D6-2C7 was a rat IgG2a, human anti-c-*fms*; Ashmun et al., 1989; 7H3, mouse IgG2a, class II; 83H1, mouse IgG1, class I and the human monocytic markers: 82H3 / CD 14, mouse IgG2a and 80H3 / CD 16, murine IgG2a; Mac Lean et al., 1982; Mannoni et al., 1982), then washed 2 times in cold PBS and incubated again for another 30 minutes under the same conditions in the presence of FITC-conjugated goat anti-rat (for the anti CSF-1 receptor antibody) and goat anti-mouse antibodies (Cappel Lab., Cochraville, PA). After washing 3 times, the cells were analyzed with an EPICS V fluocytometer (Coulter Electronics, Hialeah FL), and the fluorescent intensity of individual cells was quantified. The cells were first fixed in 2% formaldehyde because binding experiments showed that the CSF-1 receptor was unstable as a function of time (see results). Control antibodies of different specificity (for T cells) and same isotype were used: CD8 or B9.11 (mouse IgG1), CD5 or BL1 (mouse IgG2a) and CD2 or 39.C15 (rat IgG2a).

RNA extraction and Northern analysis: Total RNA from HL-60 untreated cells and T99:SS1-S2 cells (as well as BeWo and 5637 cells for

positive and negative controls) was purified by a single-step procedure by acid guanidinium thiocyanate-phenol-chloroform extraction as described elsewhere (Chomzynski & Sacchi, 1987). The RNA was then subsequently analyzed by electrophoresis of 20 ug RNA through 1% agarose-formaldehyde gel followed by Northern blot transfer to nitrocellulose (Maniatis et al., 1982). The filters were pre-hybridized at 42° C for 4 hr in buffer consisting of 50% formamide, 5X sodium chloride-sodium citrate (SSC), 0.1 % sodium dodecyl sulfate (SDS), 5X Denhardt's (a solution of Ficoll, polyvinylpyrrolidone, bovine serum albumin and water) and 200 micrograms per ml salmon-sperm DNA. The RNA blots were then hybridized as previously described (Maniatis et al., 1982) for 16 hr at 42° C using 1 x 10^6 cpm/ml of a ^{32}P-labeled 2.2 kilobases (kb) *Eco*RI fragment of human c-*fms* cDNA, cloned from a human cDNA testis bank (Clontech, Palo Alto, Ca; Dubreuil et al., 1988). After hybridization, the blots were washed twice in 2X SSC, 0.1 % SDS at room temperature for a total time of 20 minutes and then washed twice in 1X and 0.2X SSC, 0.1 % SDS at 55° C for another 20 minutes. The blots were finally dried and exposed to X-ray film (Kodak) with an intensifying screen at -70° C. The probe used (phT-cfms6) hybridizes to the 3' cytoplasmic region of the gene.

[C]. RESULTS

Ability of HL-60 cells to respond to CSF-1

The ability of HL-60 cells to respond mitogenically to CSF-1 depends upon the acquisition of CSF-1 receptors which is accomplished in two manners: 1) HL-60 cells can be induced by IFN to upregulate c-*fms* mRNA (Sariban et al., 1987) which is translated into functional CSF-1 receptors (Vassiliadis et al., 1989a); 2) T99 cells already bear CSF-1 receptors after succesful genetic manipulation using the c-*fms* gene (Sherr, personal communication). The clonogenic potential of HL-60 cells, in the presence or absence of IFN, as well as the capacity to incorporate ^{3}H-TdR in response to CSF-1 has been previously shown (chapter III). We here examine the response of T99 cells to CSF-1 for comparison to the above mentioned study.

When the T99 cells were cultured with various amounts of CSF-1 for 3 days and then ^{3}H-TdR uptake was monitored, we found that maximal (plateau) responses were reached at approximately 650 units/ml (Fig. V-1). In this study we used a CSF-1 concentration of 1,000 U/ml because it provides the same degree of mitogenic stimulation on the IFN-induced HL-60 cells (Table V-1). The ^{3}H-TdR response in both T99 and induced HL-60 cells could be specifically blocked by the anti-CSF-1 receptor monoclonal antibody 12-2D6-2C7 (Ashmun et.al., 1989) at a concentration of 0.1 micrograms of rat IgG2a protein/ml (or 1/5000; Table V-1).

Thus, although short-term treatment of IFN-induced HL-60 cells with CSF-1 and/or CSF-1 treatment of T99 cells leads to a more rapid growth, long-term treatment of both T99 and IFN-induced HL-60 cells with CSF-1 results in a decreased growth rate (Table V-3; see below).

Figure V-1

Dose response curve of CSF-1 on T99:SS1-S2 cells.

The cells were cultured with various concentrations (0 to 2,500 U/ml) of CSF-1 and 3 days later ^{3}H-TdR uptake was monitored.

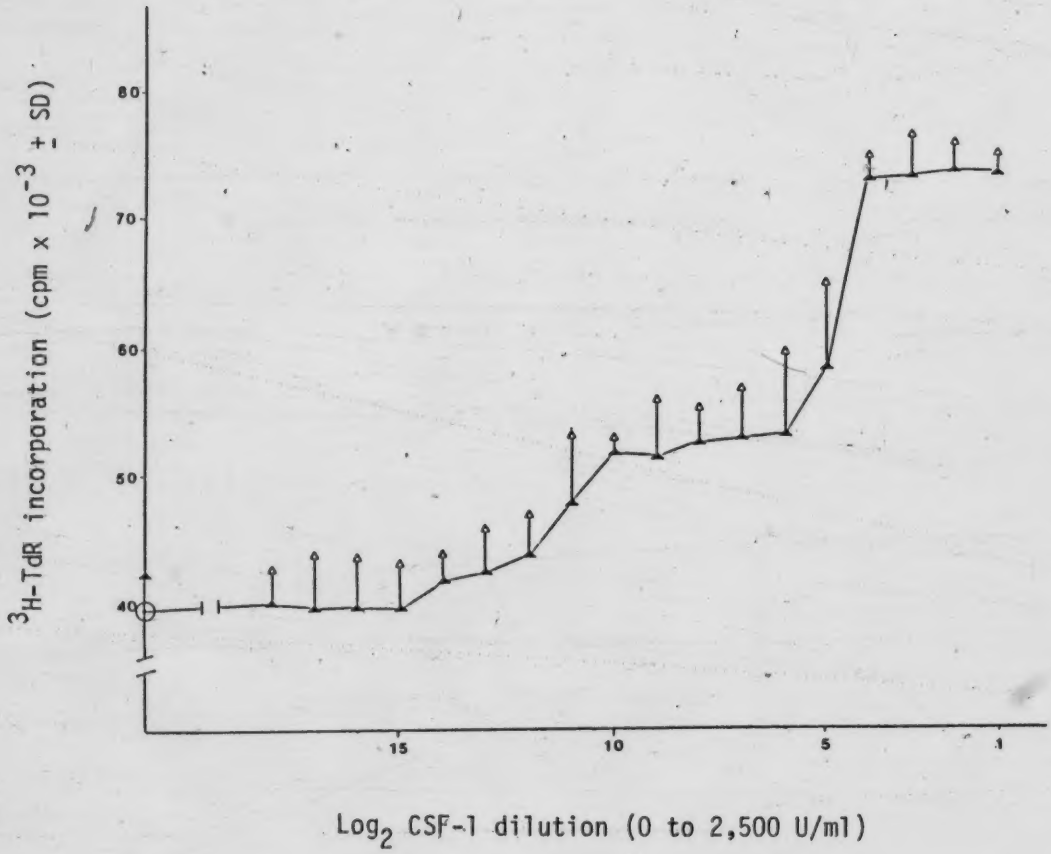


TABLE V-1

Effect of the anti-CSF-1 receptor antibody (12-2D6-2C7) on the growth of receptor-bearing HL-60 cells. The rat monoclonal antibody was added first to the T99 cultures and left with the cells for 6 hr. Subsequently CSF-1 was added at 1000 U/ml and the cultures were incubated at 37° C for 3 days until the assay day. For HL-60, the antibody was added after the IFN induction and 6 hr prior to the addition of CSF-1. Response was measured on day 10.

* The day 10 HL-60 cells were initially induced with IFN for 5 days, subsequently washed and re-cultured with CSF-1 for another 5 days.

The cells were first induced with 100 U/ml of IFN.

@ N D: not done.

& CSF-1 added at 1000U/ml and anti-CSF-1 receptor antibody 12-2D6-2C7 at the indicated dilutions that ranged from 0.1 to 1.0 ug/ml.

Treatment[&]	^{3}H-TdR incorporation (cpm ± SD)	
	T99:SS1-S2 (day 3)	HL-60 (day 10)[*]
None	46298 ± 544	71354 ± 498
CSF-1 (1000U/ml)	71245 ± 956	112816 ± 1208[#]
12-2D6-2C7 (1/500)	32523 ± 623	42588 ± 1748
" + CSF-1	32884 ± 812	45331 ± 3267
12-2D6-2C7 (1/1000)	50309 ± 1091	ND[@]
" + CSF-1	49838 ± 1196	ND
12-2D6-2C7 (1/2000)	49491 ± 899	69474 ± 1302
" + CSF-1	45318 ± 492	72263 ± 2403
12-2D6-2C7 (1/5000)	47387 ± 747	70333 ± 996
" + CSF-1	46199 ± 1463	68228 ± 1144

Binding of CSF-1 on the HL-60 cell populations; unstable receptor expression

Although we have previously shown the presence of CSF-1 receptors on IFN-induced HL-60 cells by autoradiography and binding (previous chapters), we here study the kinetics of binding on T99 cells in order to compare it with that of HL-60 and other human CSF-1 receptor positive cells.

The original HL-60 cell line after IFN treatment acquires CSF-1 receptors that bind the radiolabeled factor. Binding at 4° C, however, was detected only between 30 and 60 minutes and one hour later, the signal to noise ratio became non-significant (Fig. V-2). The same is true for the T99 cells. This observation seems to be unique for these myeloid leukemia cells (Vassiliadis et al., 1989b) and may explain the previously reported lack of correlation between expression of c-*fms* mRNA and detectable CSF-1 binding on blast cells from patients with AML.

In contrast, murine macrophages bind ^{125}I-CSF-1 irreversibly at 4° C (Guilbert & Stanley, 1986) Human normal monocytes, placental and choriocarcinoma cells (BeWo; Rettenmier et al., 1986) exhibit the same degree of irreversible binding like the murine cells under the same experimental conditions (Lloret & Guilbert, unpublished).

Percentage of cells expressing the receptor

Thus, quantitation of receptor numbers required that receptor detection be carried out at early times. For this, the cells were first fixed in

Figure V-2

Binding of ^{125}I CSF-1 on T99:SS1-S2 and HL-60 cells over time.

The experiment was carried out at 4° C as described in the Methods. Both kinetic curves of total binding shown here represent the mean of two experiments carried out in triplicates. The solid lines represent binding of the constitutively CSF-1 receptor-bearing cells whereas the broken lines show binding of HL-60 cells. The vertical bars show the amount of non-specific binding.

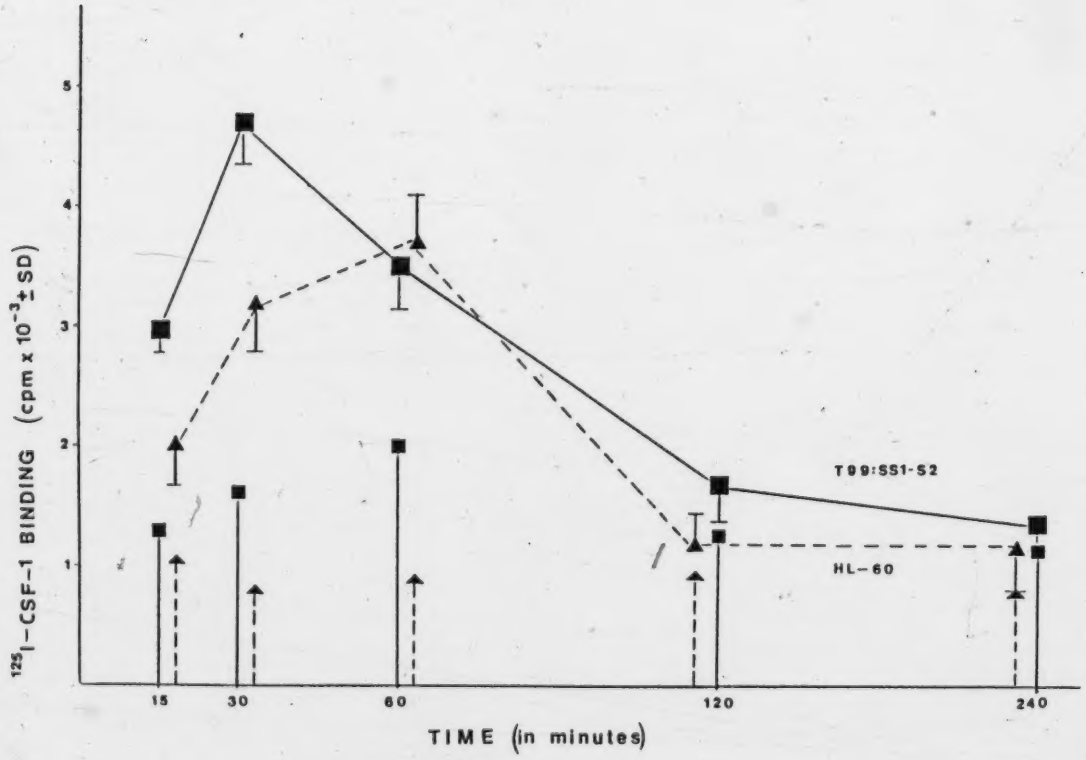


Figure V-3

FACS analysis of the presence of CSF-1 receptor on the surface of fixed c-*fms*-positive cells. The upper histogram shows the percentage of CSF-1 receptor-negative cells when stained with PBS, whereas the lower histogram shows the cells that have been tagged with 0.5 ug/ml of the anti-c-fms monoclonal antibody. The test was carried out in the dark at 4° C.

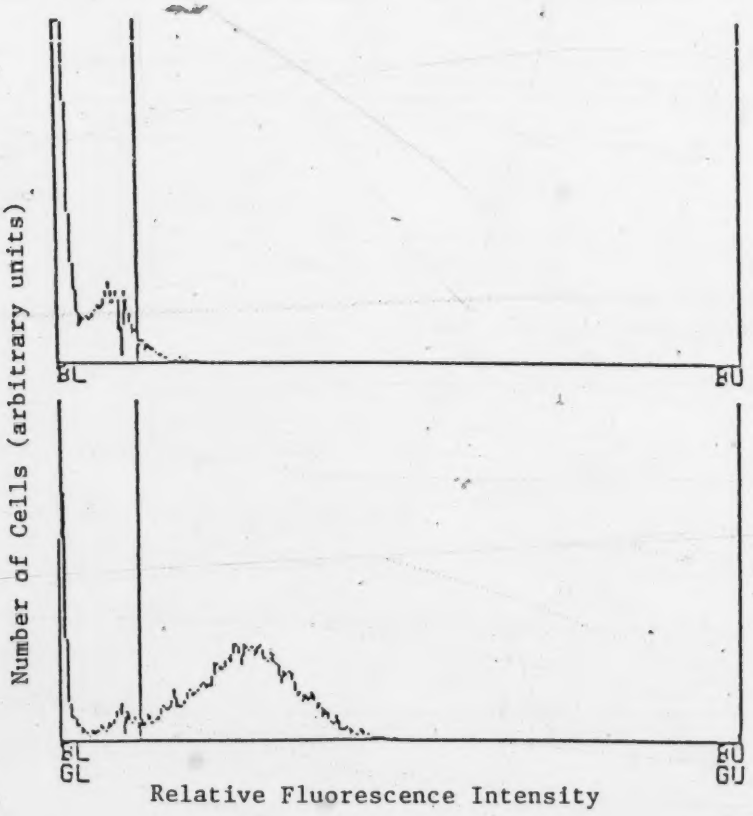


TABLE V-2

Marker analysis of 2% formaldehyde-fixed T99 and HL-60 cells.
T99 cells were taken out of the culture and tested for surface marker expression on day 0. However, HL-60 cells were first induced with IFN for two days and then maintained in culture for the full 10 day period before the analysis. In both cases the cells were processed as described in the Methods.

	% of positive cells by FACS	
Antibodies (specificity)	T 99	HL-60*
PBS (negative control)	4	8
83H1 (class I)	90	99
7H3 (class II)	18	10
82H3 (CD14)	52	14
80H3 (CD16)	5	23
12-2D6-2C7 (1/1000; anti-c-fms)	64	15

* The class I, class II, CD 14 and CD 16 markers are maximally induced on day two after IFN treatment and they decline to pre-induction levels by day 10.

2% formaldehyde and then analyzed for surface marker expression all within 30 minutes. We found that 64% of the T99 cells were receptor positive by using the rat monoclonal antibody 12-2D6-2C7 (Fig. V-3). A detailed analysis of this and other surface markers is given in Table V-2.

As Table V-2 shows, the IFN-induced HL-60 cells did not show a significant degree of cell-surface associated CSF-1 receptor. This can be explained by the receptor-density difference between the constitutively expressing c-*fms* T99 cells and the IFN-induced population. We have previously shown that only 33 to 37 % of the HL-60 cells can be induced to express the CSF-1 receptor after IFN treatment (previous chapters). In addition, Sariban et al. (1987) has demonstrated that IFN induces the expression of a moderate amount of c-*fms* mRNA in HL-60 cells. These two observations taken together may explain why induced HL-60 cells have a lower surface expression of c-*fms* as shown in Table V-2. In contrast , *de novo* synthesis of c-*fms* mRNA can be demonstrated on T99 cells by Northern blot analysis (Fig. V-4). mRNA was isolated (Chomzynski & Sacchi, 1987; Maniatis et al., 1982) and subsequently hybridized with a 2.2 kb fragment of human c-*fms* cDNA probe recognizing the 3' end of the gene as described in detail in the Methods (Dubreuil et al., 1988). Exposure of the nitrocellulose paper on X-ray film showed the presence of c-*fms* on the T99 cells, indicating constitutive expression of the CSF-1 receptor (Fig. V-4). In contrast, untreated HL-60 cells did not show any message.

Effect of CSF-1 and IFN on CSF-1 receptor positive HL -60 cells

HL-60 cells, which showed responsiveness to CSF-1 after IFN induction, were maintained in culture for an extended period of time in the

Figure V-4

Identification of c-*fms* mRNA by Northern blot analysis:

20 micrograms of total RNA were run on a denaturing agarose gel and transferred onto a nitrocellulose paper. The filter was hybridized with a 2.2 kb human c-*fms* probe labeled with ^{32}P-dCTP by random oligonucleotide priming. Each lane represents RNA isolated from: 1, 5637 cells (human bladder carcinoma-negative control); 2, BeWo cells (human choriocarcinoma -positive control); 3, HL-60 cells; 4, T99:SS1-S2 cells; 5, BeWo cells. The RNA from lanes 3,4 and 5 was isolated according to the single step purification method as described by Chomczynski & Sacchi (1987), whereas the RNA from lanes 1 and 2 was isolated by the method of Maniatis et al., (1982).

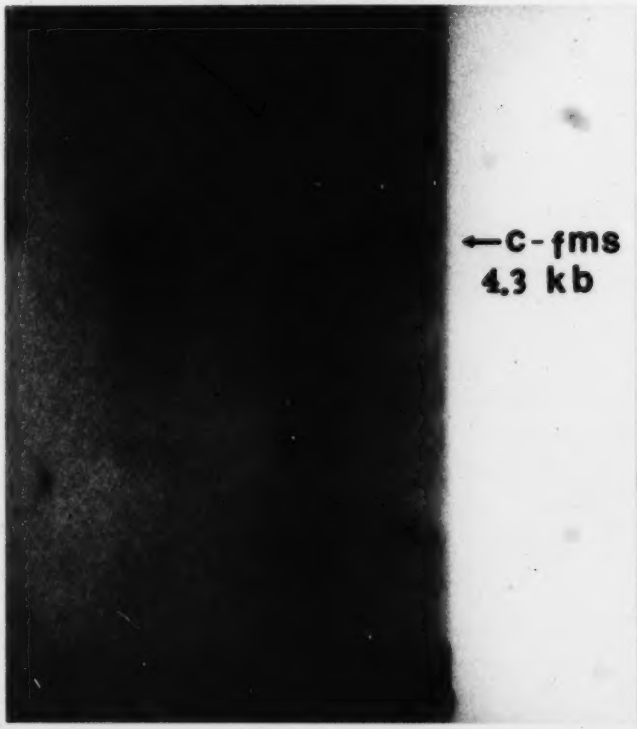


TABLE V-3

Long-term effect of CSF-1 and IFN on CSF-1 receptor positive (T99:SS1-S2) and negative (HL-60) cells. The cells were fed and maintained in culture in the presense of the factors indicated. Tests were performed after a period of 20 days and two months, respectively. At regular time intervals, 5000 cells were re-cultured per well in the presence of IFN, CSF-1 or both. Three days later ^{3}H-TdR uptake was monitored. The experiments shown here are representative of a series of at least 10 assays carried out in triplicates.

T99:SS1-S2:

Treatment	^{3}H TdR incorporation (cpm $\pm$ SD)	% suppression
none	48768 $\pm$ 1170	0
IFN	32161 $\pm$ 1371	34.0
CSF-1	33236 $\pm$ 648	32.0
IFN + CSF-1	26505 $\pm$ 990	46.0

HL-60:

none	56824 $\pm$ 325	0
IFN	46118 $\pm$ 524	18.0
CSF-1	51731 $\pm$ 639	10.0
IFN + CSF-1	39202 $\pm$ 582	31.0

presence of either IFN or CSF-1 or both. The same culture conditions were followed for the T99 cells as well. In an attempt to study the role of CSF-1 on these leukemic cells, 5000 cells were taken out of the bulk culture, washed and re-cultured with and without CSF-1, IFN or both and their ability to incorporate ^{3}H-TdR was tested 3 days later.

For twenty and sixty days the T99:SS1-S2 and the induced HL-60 cells respectively were able to respond to CSF-1 as measured by increased incorporation of ^{3}H-TdR. However, after this time-period, we observed that CSF-1 began to suppress the proliferation of the cells (Table V-3) as did IFN alone. Surprisingly, when both cell populations were exposed to the combination of IFN plus CSF-1 the growth inhibition was more profound (see below) despite the fact that the cells were able to bind ^{125}I-CSF-1 (364 ± 86 versus 1520 ± 82 cpms for the T99 cells and 243 ± 66 versus 972 ± 77 cpms for the HL-60 population).

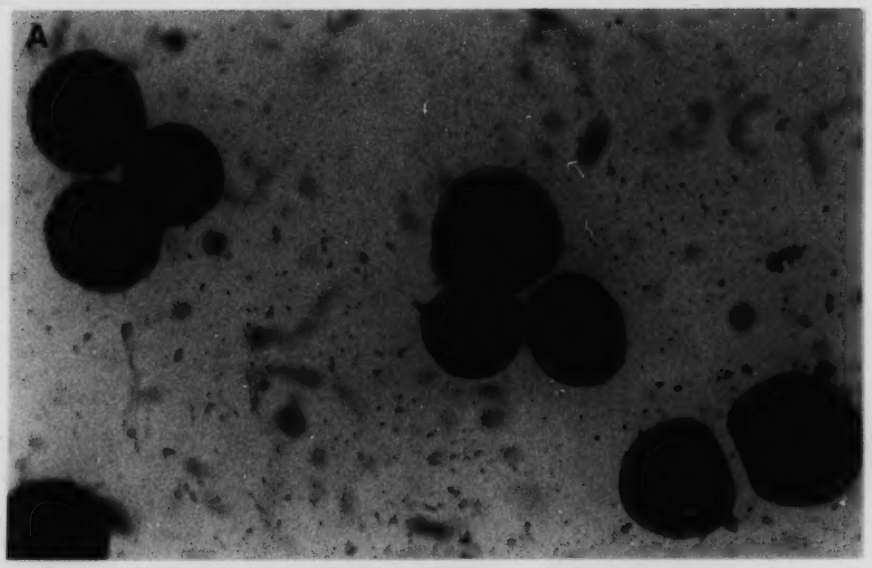
The combination of IFN + CSF-1, after the same prolonged treatment period, drove four out of nine T99 cultures to stop growing, indicating that the combination of both factors acted to arrest the growth of T99 cells. Three out of seven HL-60 cultures responded in a similar way. In addition, morphological studies showed that the combination of the two factors resulted in cells with a complete myeloid appearance, i.e., presence of cells with myeloid characters including a highly granulated cytoplasm indicative of basophiles and eosinophiles (Fig. V-5). CSF-1 alone was not able to induce any morphological differentiation, whereas IFN alone induced monocytic differentiation.

The mean percentage of cells with normal morphology was estimated to be between 30 and 65% per slide or per culture. Removal of the

IFN/CSF-1 combination resulted in cell death in cultures with a high differentiated-to-non differentiated cell ratio, whereas in cultures with a low ratio of differentiated cells an increased cell accumulation was observed accompanied by high ^{3}H-TdR uptake approaching the control levels. Removal of either IFN or CSF-1 resulted in increased cell accumulation. A summary of these results is given in Table V-4 where cell numbers / ml of culture have been monitored before and after the removal of IFN, CSF-1 or both. These results demonstrate that at least a fraction of cells bearing the CSF-1 receptor can be driven under normal control after appropriate treatment with the combination of IFN and CSF-1.

Figure V-5

Morphological changes accompanied by long-term treatment of IFN and CSF-1. The cells were stained with Wright's-Giemsa and examined under 100x. **A:** HL-60 control untreated cells; **B:** IFN treated cells.



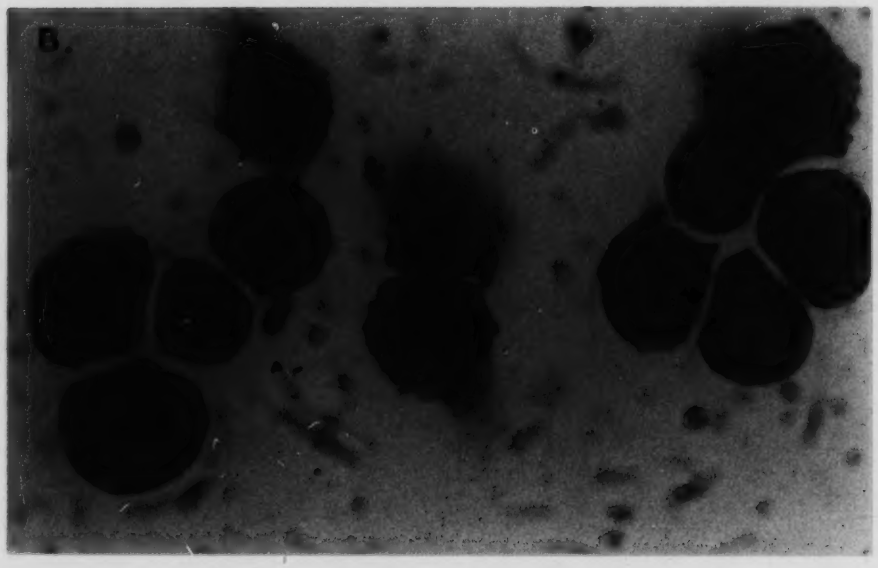
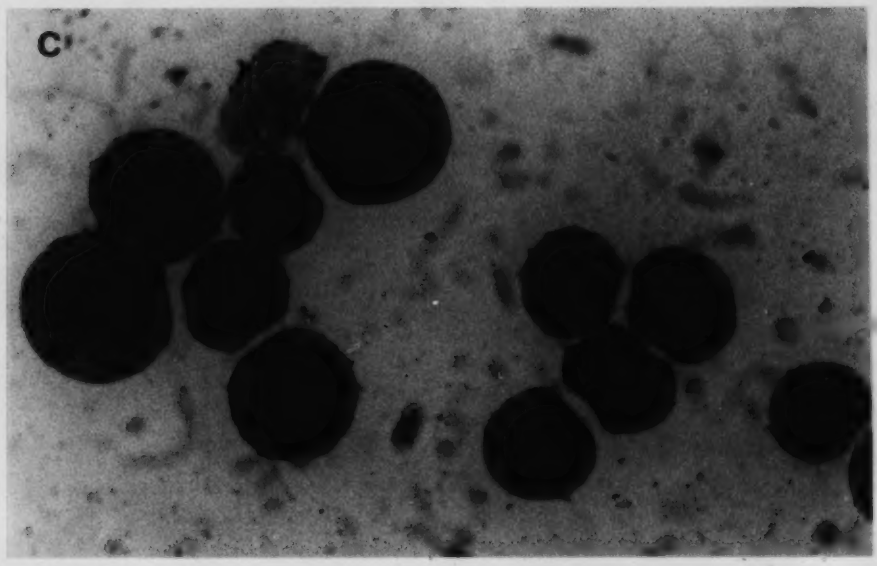


Figure V-5, cont'd

C: CSF-1 treated cells and D: cells exposed to the combination of IFN and CSF-1.



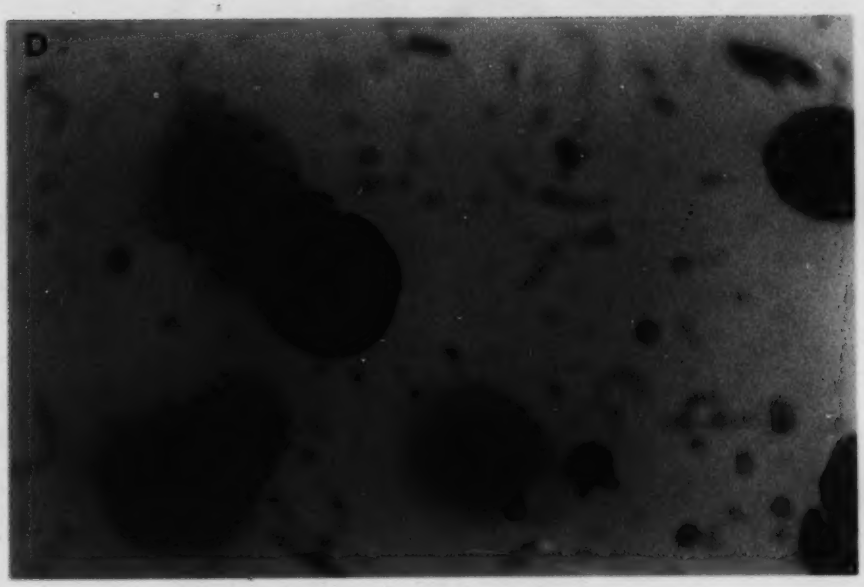


TABLE V-4

Cell-growth pattern of the long-term treated cells after removal of IFN, CSF-1 and the combination of both.

	Number of viable cells per ml of culture ($x10^{-6}$)#			
	T99:SS1-S2		HL60	
Factor removed	A*	B&	A	B
None	0.5	0.5	0.5	0.5
IFN	0.7	0.8	1.0	0.8
CSF-1	0.6	0.8	1.25	0.75
IFN+CSF-1	1.1	0.02	1.25	0.13

The indicated number of cells ($0.5x10^6$) was taken out of the original long-term CSF-1+IFN containing culture, washed and subsequently cultured in a fresh dish without the indicated factor. Three days later, viable cell count was determined by trypan blue dye exclusion and compared to the original seeding numbers.

* A: Cultures with a low differentiated-to-non differentiated cell ratio (<50%).

& B: Cultures with a high differentiated-to-non differentiated cell ratio (>60%).

[D]. DISCUSSION

CSF-1, the mononuclear phagocyte-specific factor (Kawasaki et al., 1985; Stanley et al., 1982; Guilbert, 1985), supports the proliferation, differentiation and survival of murine macrophages and macrophage precursors (Byrne et al., 1981; Tushinski et al., 1982; Guilbert, 1985; Guilbert & Stanley. 1986). In the human system, however, little is known about its actions. It increases the proliferation of certain cells bearing the CSF-1 receptor including choriocarcinoma (BeWo; Rettenmier et al., 1986), placental and amniochorion cells (Bulmer & Johnson, 1984; Pollard et al., 1987 & 1989), activates macrophages (Nathan et al., 1984), and promotes the proliferation of some AML cells either at the blast or at a more mature stage (Dubreuil et al., 1988; Miyauchi et al., 1988; Pèbusque et al., 1988).

CSF-1 does not have well established differentiation capabilities on human cells. Such an action has been described only for a certain (sub)population of AML blasts which show increased adherence accompanied by some surface differentiation characteristics (Miyauchi et al., 1988). In addition, CSF-1 has no known synergistic actions with other growth factors in this respect.

In order to study the role of CSF-1 on human leukemia, we here used the human promyelocytic leukemia HL-60 (Collins, 1987) which can be induced to differentiate towards the monocytic pathway (Ameglio et al., 1983; previous chapters). Upon induction with IFN, the cells transiently resemble their normal counterparts by expressing a number of common normal surface antigens (Rosa & Fellous, 1984; Collins, 1987), as well as the

mRNA for c-*fms* (Sariban et al., 1987), which is translated to a functional CSF-1 receptor (Vassiliadis et al., 1989). We also employed a HL-60 variant (T99:SS1-S2; Sherr, unpublished) expressing constitutively the CSF-1 receptor (Figures V-2, -3, -4 and Table V-2).

We found that both cell populations, either induced to express the CSF-1 receptor or constitutively expressing the receptor, were responsive to CSF-1 as monitored by incorporation of ^{3}H-TdR for a relatively short time (Fig. V-1 and previous chapters). However, after prolonged exposure (more than 20 days for T99 and 60 days for HL-60) of the cells to CSF-1, cell growth slowed. As Table V-3 demonstrates, CSF-1 started having a suppressive effect on cell growth after a long-term treatment of the IFN-induced HL-60 (10%) and T99 (32%) cells although both cell sets maintained their ability to bind radiolabeled CSF-1. The combination of IFN plus CSF-1, however, gave the strongest results. By treating the cells (both T99 and induced HL-60) with IFN + CSF-1, after 20 and 60 days respectively, we found that in approximately 40% of the cultures the cells were unable to proliferate. IFN alone maintained its known capacity to suppress the growth of the cells. However, IFN in combination with CSF-1 gave a much potent growth inhibitory signal (Table V-3).

The difference in timing (20 versus 60 days) between the two HL-60 populations may be explained by the CSF-1 receptor density which is being maintained constant for T99 cells (Fig. V-4). Removal of both CSF-1 and IFN from the long-term treated cultures resulted in cell death (Table V-4). Inability of growth was noted especially in cultures where the differentiated-to-non differentiated cell ratio was greater than 60%.

IFN has been reported to induce monocytic differentiation (Collins, 1987). This effect, however, has been shown to occur only after a 5-day

treatment (Collins, 1987). In this work, we confirmed the transition of HL-60 cells from myeloblasts to monocyte-like cells even after a prolonged exposure to IFN (Fig. V-5). In combination with CSF-1, however, a more complete differentiation signal was delivered to the cells that resulted in the generation of a population with visual myeloid characteristics.The differentiated cells (Fig. V-5) exhibited a hypergranular cytoplasm stained by Wright's-Giemsa black and red, indicating basophilic and eosinophilic differentiation. Whether these cells are representative of the myeloid lineage remains to be determined.

This is the first indication of a synergistic effect between CSF-1 and IFN on CSF-1 receptor positive cells. The findings suggest that it is possible to induce malignant cells to a normal phenotype. They also suggest that the presence of CSF-1 receptor may be crucial in the process of differentiation. The real potential of CSF-1 as a differentiation agent may be demonstrated when in combination with other factors like IFN. CSF-1 probably needs to synergize with other biological regulators in order to bring the abnormal pathophysiology of malignant AML cells under normal regulatory mechanisms. Clonal extinction greatly depends on the appropriate signal transduced that, in this HL-60 case, appears to be CSF-1-mediated.

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CHAPTER VI

DISCUSSION

[A]. GENERAL DISCUSSION

The main objective of this research was to develop an experimental model and use this to determine whether colony-stimulating factor-1 (CSF-1) operates on leukemic cells bearing receptors for this factor, by bringing them to a controlled differentiated state. This work was undertaken to contribute to leukemia research, especially the control of unregulated cellular proliferation in acute myeloid leukemia (AML).

CSF-1 is a potential inducing agent for AML cells, able to promote proliferation and differentiation. Although neither signal is well defined, the observed proliferation (Pébusque et al., 1988) is assumed to be due to interaction between CSF-1 and its receptor. The latter appears to be present in all primary AML populations; i.e., they express the proto-oncogene c-*fms*, which encodes the CSF-1 receptor (Dubreuil et al., 1988). However, the percentage of cells in a grown population that express functional receptors is not known.

The assumption that all AML cells bear CSF-1 receptors holds for the differentiation signal(s) also. Although it is widely believed that the receptor plays an important part in differentiation, the factor (CSF-1) appears to affect only some of the receptor-bearing AML blast cells, inducing them to a normal, functional phenotype (Miyauchi et al., 1987 & 1988). The criteria for selection of only some of the c-*fms*-positive AML cells are not known, a

problem based in the heterogeneity of the primary AML cell population (reviewed by Andreeff & Welte, 1989): the rapid proliferation of cells that receive signals to divide could mask timing, direction, and patterns of other complex events. Thus, investigating the role of CSF-1 in the differentiation of leukemia cells requires use of a model containing CSF-1-receptor-positive leukemia cells (like primary AML) that are homogeneous as a population (unlike primary AML).

One cell line (HL-60) satisfies the latter criterion (Collins et al., 1977). It also conforms to the former criterion after induction with immune interferon (IFN; Sariban et al., 1987; Vassiliadis et al., 1989). We therefore used this cell line in developing a model to study how IFN up-regulates the CSF-1 receptor and then to investigate the effect of CSF-1 on a homogeneous population of leukemic cells representing an AML-M2 subtype (Dalton et al., 1988).

IFN has a strong potential for cellular differentiation. After being in culture for several days, it can induce HL-60 cells to differentiate towards the monocytic pathway, as shown by analysis of cell-surface markers, phagocytosis, and morphology (Collins et al., 1977; Ameglio et al., 1983; Becker, 1984; Collins, 1987; chapter III). In addition, IFN induces expression of mRNA for the molecular markers c-*fms*, c-*fos*, TNF, and class II, and of the monocytic marker interleukin-2 (IL-2) receptor (Sariban et al., 1987 & 1988; Herrmann et al., 1985; chapter II).

1. IFN up-regulates functional CSF-1 receptors

This knowledge led to the first hypothesis tested in this study: *That IFN can induce HL-60 cells to express functional CSF-1 receptors during monocytic differentiation;* i.e., it was questioned whether IFN can up-regulate the

receptor following induction of the c-*fms* mRNA (Sariban et al., 1987).

As IFN induced the HL-60 cells to mature along the monocytic pathway, binding studies revealed functional CSF-1 receptors on about 35% of the total population (chapter III). This expression was concomitant with the appearance of c-*fms* mRNA (day 7 and beyond), confirming findings in kinetic studies by Sariban et al. (1987). Expression of the receptor did not require the continuous presence of IFN, being unaltered on days 10 to 14 by IFN's removal from the cultures on day 8 or 10 (chapter III). Also, once the HL-60 cells had been induced by IFN to differentiate, they did not revert to their receptor-negative state for at least 14 days. The use of various induction protocols to define the exact timing of IFN's action on HL-60 cells showed that induction of CSF-1 receptors never occurred before a 2-day treatment and always by day 8. This differs from most IFN-mediated events, which usually occur within 48 hr; e.g., the expression of HLA-DR, the surface human leukocyte antigen-DR (Ameglio et al., 1983). For example, IFN-induced DR expression in HL-60 cells was transient, peaking on day 2 and then dropping to pre-induction levels by day 7-8 as expression of CSF-1 receptors became manifest (chapter IV).

This inverse relationship between the two markers appears to be mechanically related. HLA-DR was chosen for study, in preference to markers such as HLA class I or IL-2 receptor, because most CSF-1-responsive cells are DR-positive (Springer et al., 1979); also, IFN-induced HLA-DR expression is mediated by the Ca^{2+}/Calmodulin pathway (Ina et al., 1987; chapter IV), rendering the system amenable to analysis.

The different times of IFN action, early and late, have not been explained. Therefore, HLA-DR versus CSF-1 up-regulation of receptors was studied, to determine whether early events represented an initial step in the

induction of differentiation, requisite to maturation events later. Induction of the cells with IFN and sorting of the resultant DR-positive population, showed that the cells which expressed high levels of DR were the ones that up-regulated the receptor later in culture (chapter IV). This was further studied to confirm that DR could predict expression of the CSF-1 receptor by preventing cells from expressing DR.

Theophylline (TPH) blocks IFN's action on class II expression by specifically inhibiting the interaction between calmodulin and phosphodiesterase (Ina et al., 1987; Means & Dedman, 1980). In the present study, TPH suppressed CSF-1 receptor up-regulation and responsiveness when applied before the induction of class II antigen (chapter IV). In addition, complement-mediated lysis of DR-positive cells, after IFN treatment, eliminated cells later able to respond to and bind CSF-1. These results indicate that IFN-induced class II expression predicts up-regulation of CSF-1 receptors.

Although IFN-induced expression of class II antigen could predict up-regulation of CSF-1 receptors, HLA-DR by itself could not predict the CSF-1 response; calcium ionophore A23187, another agent that can induce HLA-DR expression (Ina et al., 1987), could not up-regulate the CSF-1 receptor (chapter IV). Only IFN-induced class II-positive cells developed the capacity to become CSF-1 responsive later, confirming the importance of IFN in monocytic differentiation of HL-60 cells.

HL-60 cells, constitutively positive for DR, were unresponsive to IFN (chapter IV). These cells could not up-regulate CSF-1 receptors and therefore did not respond to CSF-1. One possibility for this behavior is that the cells had to go through an HLA-DR cycle after IFN treatment in order to

acquire the receptor, suggesting that cells which were already DR-positive had missed their timing in differentiation and could not re-enter the pathway. Sorting experiments clearly demonstrated that DR-positive cells are resistant to the action of IFN and cannot be induced to express CSF-1 receptors indicating that the above possibility may be viable. Only class II-negative cells were sensitive to IFN's action: they could be induced to express the surface antigen HLA-DR on day 2 and to become CSF-1 responsive later in culture (chapter IV).

Thus, the data indicated that induced HLA-DR expression was a parallel event, not a causative one, in up-regulation of the CSF-1 receptor. As IFN targeted a specific subpopulation of HL-60 cells (about 80% from which 35% expressed the receptor; chapters III & IV), it seems that even a well-characterized cell line (i.e., HL-60) can give rise to problems of heterogeneity. The cells' heterogeneity was confirmed in autoradiographic studies, in which only 33-37% of the induced cells developed the ability to acquire CSF-1 receptor and respond to CSF-1 (chapter III).

Although the expression of CSF-1 receptors had been established, the response to CSF-1 (or the binding) after IFN induction could have been the indirect effect of an intermediate cytokine produced during the treatment and the production of hemopoietins from cell-to-cell interactions could have stimulated cellular proliferation directly. These possibilities were examined.

IFN's effect in up-regulating the CSF-1 receptor was independent of, at least, interleukin-1 (IL-1) and tumor necrosis factor (TNF). These two cytokines synergize with IFN on HL-60 cells: IFN and IL-1 act on human monocytes and HL-60 cells to promote cell growth (Newton, 1985; Tsai & Gaffney, 1987). TNF combined with IFN has a more potent differentiation effect than either alone (Trinchieri et al., 1986). Thus, as IFN treatment of

HL-60 cells induces the expression of mRNA for TNF (Sariban et al., 1987), TNF produced during the IFN treatment could synergize with or potentiate the effects of IFN in the process of up-regulation of receptors.

Secreted TNF is a polypeptide hormone composed of identical 17 Kd subunits arranged in a dimeric, trimeric or pentameric form depending upon species and method of isolation (Oettgen & Old, 1987). There is no firm evidence that the protein is glycosylated in any species, although the mouse protein sequence contains potential glycosylation sites (Aggarwal et al., 1985; Beutler & Cerami, 1986). Another TNF form (26Kd) has been reported that is membrane-associated (Beutler & Cerami, 1986).

The main physiologic function of TNF is not known. It holds promise, however, as an antineoplastic agent, because it greatly impairs the *in vitro* proliferation of several types of cells and affects the differentiation of others (Trinchieri et al., 1986).

Concomitant with the IFN-induced mRNA for TNF were the induction of HLA-DR (chapter IV) and enhanced expression of HLA class I (Vassiliadis, 1985). This concomitant expression of TNF message and surface-associated HLA seems to be a logical consequence, as the genes for these markers reside on the same chromosome: in humans the TNF gene is on chromosome 6 and is HLA-linked, whereas in mice the gene is on no. 17 within the D region of the H-2 complex (Old, 1985; Beutler & Cerami, 1987). However, functional expression of protein in IFN-induced HL-60 cells occurs only with HLA-DR.

Soluble TNF production that could influence the results towards CSF-1 responsiveness was excluded by experimentation with multiple washings after induction, to eliminate any TNF or IL-1 that might have been secreted

or had accumulated. After IFN treatment and multiple washings, the HL-60 cells retained their ability to respond to and bind CSF-1, as expected (chapter III), ruling out the possibility of synergy. The results also indicated that no other secreted factor was participating in the IFN-induced up-regulation of the CSF-1 receptor. However, the experimental design had not excluded the possibility of effects of a surface-associated factor.

We find that TNF at all concentrations inhibits, rather than stimulates, CSF-1-stimulated proliferation by directly bringing HL-60 cells to a relatively mature, CSF-1 receptor-expressing but nongrowing state (chapter III). Thus, involvement of surface-associated TNF as an intermediate (in the IFN induction process) to a morphologically immature CSF-1 receptor-expressing and growth competent state appears unlikely.

It was noteworthy that not all mRNA detected after IFN induction translated into a functional protein product: c-*fms* mRNA was translated to a functional receptor, but TNF's message was not. No TNF was detected on bioassays, in which 50 pg/ml is the lowest level of detection, indicating no translation of mRNA.

Also noteworthy is the observation that TNF can induce the expression of CSF-1 receptors on HL-60 cells, of the same magnitude as IFN (chapter III), but resulting in a different functional state: IFN-induced receptors were able to transduce mitogenic signals, whereas TNF-induced receptors were unable to. This raises questions about the degree of differentiation induced by the two factors. TNF more rapidly and completely induces differentiation than IFN does (Beutler & Cerami, 1986). Expression of CSF-1 receptors and a nongrowing cell-state are properties of differentiation of normal monocytes, whereas receptor-expression and growth competence is a transient property occurring probably near the

monoblast stage (van Furth, 1981). Because TNF also stimulates expression of CSF-1 mRNA in HL-60 (Sherman et al., 1989), an intriguing possibility is that the combination of CSF-1 and its receptor drives differentiation to nongrowing state. However, the timing of TNF-induced expression is not appropriate [CSF-1 rises and falls before receptor rises (Sherman et al., 1989; chapter III)]. We cannot detect TNF protein after IFN induction (chapter III) and we find that interaction of CSF-1 with its receptor on HL-60 cells is not sufficient to bring the cells rapidly to a mature nongrowing state (chapter V).

2. Investigation of the role of CSF-1 on the leukemic cells

The second hypothesis of this work was *that CSF-1 can act through its IFN-mediated receptor to trigger a differentiation signal to initiate maturation, which can lead a leukemic clone to proliferative senescence and even extinction.* To study this, the required homogeneous population of cells expressing the CSF-1 receptor could not be isolated because CSF-1 receptors were probably shed very rapidly from the surface of the induced HL-60 cells (chapter V; see below). To circumvent this problem, a variant of the HL-60 cell line (T99:SS1-S2) was characterized that express constitutively CSF-1 receptors. T99 cells were obtained by retroviral infection, using a c-*fms* neoresistance-containing vector packaged in PA317 cells. After selection in G418, the cells were sorted twice with antibody to CSF-1 receptor, to generate the receptor-positive population (Sherr; personal communication).

CSF-1 triggered these cells to proliferate in a similar manner as with IFN-induced HL-60 cells (chapters II to V) but without inducing differentiation. Thus, CSF-1 did not rapidly induce differentiation to a nongrowing state. This result raised several questions. Was the proliferation

a necessary step in the overall differentiation action of CSF-1? Was CSF-1 helping the cells to complete the number of cell divisions required to enter the differentiation process?

To answer these questions, long-term studies were performed in which cell behavior was examined after several cycles . Prolonged treatment with CSF-1 suppressed proliferation, indicating that differentiation was possible, but morphologic examination of the treated cells revealed absence of maturation (chapter V). This limited effect of CSF-1 in differentiation indicated that CSF-1 may have required "help" from another inducing agent. In fact, prolonged treatment with IFN and CSF-1 combined slowed the growth of the cells and shifted their morphology toward a more differentiated myeloid state (chapter V). Induced HL-60 as well as T99:SS-S2 cells behaved similarly.

Although IFN alone suppressed growth and CSF-1 augmented proliferation at the beginning of the cultures, prolonged treatment with IFN and CSF-1 combined completely stopped the growth of up to 40 % of the cultures. The proliferative senescence was accompanied by large numbers of differentiated myeloid cells, as assessed by morphology (chapter V), including highly granulated cells with mature eosinophilic and basophilic characteristics. Cells obtained after treatment with IFN plus CSF-1 were not hypersegmented end-stage cells like those that exist in the peripheral blood of healthy persons. However, they were well beyond the blast and/or promyelocyte stage that predominate in the HL-60 untreated population (chapter V); perhaps they represent end-stage cells for this AML-M2 disease and this is a matter that deserves further investigation.

It thus appears that CSF-1 alone has a limited role in the

differentiation of leukemic cells in humans. When combined with IFN, however, it induces relatively complete myeloid maturation, as demonstrated by morphologic changes and senescence (chapter V). Why the myeloid pathway is preferentially selected over the monocytic one is not known. IFN by itself induces HL-60 cells to monocytes, but when combined with CSF-1 it induces differentiation into basophils and eosinophils.

One very interesting aspect of the induced or constitutively expressed receptor on HL-60 was that it did not behave like its murine-macrophage counterpart. Both murine and human CSF-1 bind irreversibly and stably to the mouse receptor at 4° C, but at 37° C, binding is reversible and characterized by rapid internalization followed by factor degradation. The binding reaction of human CSF-1 to the induced human receptor on HL-60 cells could not be reproducibly carried out using the mouse conditions and detection of the receptor by direct binding studies with ^{125}I-CSF-1 at 4° C was difficult. The time course studies (chapter V) demonstrated that binding was maximal at 30 to 60 minutes and decreased to background levels after two hours. The same results were obtained with the HL-60 variant, T99:SS1-S2. However, contrasting results were obtained with human normal monocytes, placental cells or human choriocarcinoma BeWo cells where ^{125}I-CSF-1 bound irreversibly and stably (Lloret & Guilbert, unpublished). Flow cytometric analysis indicated that the cell surface expression of epitopes recognized by a receptor specific monoclonal antibody were more stable on fixed than unfixed T99 cells (Vassiliadis et al., 1989b). These observations suggested that both unoccupied and occupied CSF-1 receptor is rapidly lost from the surface of the human leukemia cells at 4° C and may explain the previously reported lack of correlation between expression of c-*fms* mRNA and detectable CSF-1 binding on blast cells from patients with AML

(Nienhuis et al., 1985).

This property of the human CSF-1 receptor may indicate vital differences in signal transduction requirements of leukemic cells compared with normal monocytes or mouse cells. Rapid self-internalization and/or shedding may modulate signal transduction to the cell nucleus, each perhaps acting independently. Especially in AML, where almost all cells express mRNA for the CSF-1 receptor (Dubreuil et al., 1988), reducing the numbers of receptors may influence the outcome of the disease. Alternatively, rapid shedding may prevent adequate differentiation signaling. This is in addition to the functional state of the CSF-1 receptor (IFN versus TNF; chapter III), which may determine predisposition to the AML disease.

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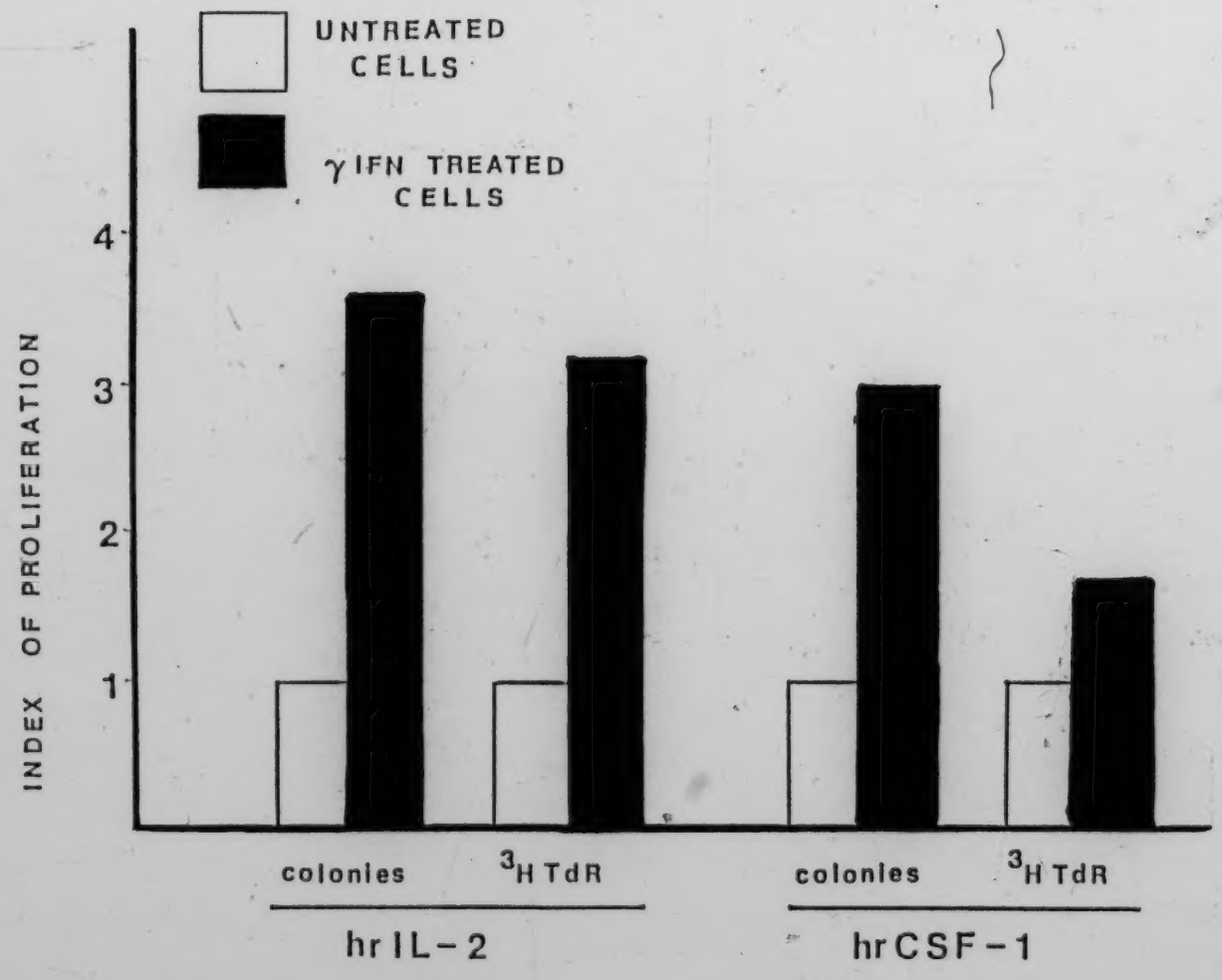
APPENDIX 1

^{3}H-TdR incorporation assays as well as clonogenic assays in methylcellulose demonstrate that IFN treatment of HL-60 cells is up-regulating response to IL-2 and CSF-1. Detailed description of the assays is provided in the Methods of chapter III.



APPENDIX 2

Effect of theophylline (TPH) on IFN-induced expression of cell-surface antigens on HL-60 cells after 48 hr of treatment. The cells were treated and analyzed as described in detail in the Methods of chapter IV.

	Percent of positive cells*				
Culture components	PBS	class I	class II	CD 14	CD 16@
Nothing	10	98	16	8	24
IFN	5	91	52	31	56
TPH	3	94	22	28	50
TPH + IFN	2	95	22	31	49

* Determined by indirect immunofluorescent analysis on FACS.

@ The monoclonal antibodies used were: 83H1 (IgG 1) for class I, 7H3 (IgG 2a) for class II, 82H3 (IgG 1) for CD 14, 80H3 (IgG 2a) for CD 16 and PBS as negative control.

APPENDIX 3

Effect of 0.18 mg/ml theophylline on IFN-induced up-regulation of functional CSF-1 receptors on IFN-treated HL-60 cells.

Culture components			^{3}H-TdR incorporation	CSF-1 Receptors/cell(±SD)#
CSF-1	IFN@	TPH	(cpm±SD)	
--	--	--	52542 ± 381	437 ± 54
+	--	--	54016 ± 476	250 ± 22
--	+	--	45323 ± 381	1618 ± 77
+	+	--	59586 ± 222	1347 ± 68
--	--	+*	30556 ± 986	132 ± 15
+	--	+*	32034 ± 497	113 ± 20
--	+	+*	24854 ± 468	187 ± 65
+	+	+*	22756 ± 905	276 ± 98
+	+	+&	58541 ± 123	1224 ± 106
--	--	+&	49877 ± 305	205 ± 55
--	+	+$	26556 ± 408	1176 ± 144
--	--	+$	33478 ± 654	167 ± 97

@ Where indicated IFN was present throughout the culture at 100 U/ml.

* TPH was added from the beginning of the culture

& TPH was added one day before the test

$ TPH was added after the 5-day induction period

Numbers of CSF-1 receptors/cell determined from specific binding on day 10.

APPENDIX 4

HL-60 supernatants tested on TNF-sensitive and -resistant cell lines reveal no TNF production before or after IFN treatment.

	Optical Density (±SD) on:	
Factor or Sup.	L929.8 (TNF-sensitive)	L929-R (TNF-resistant)
---	0.468±0.016	0.503±0.015
TNF (5 ng/ml)	0.296±0.003	0.485±0.018
Untreated HL-60	0.417±0.010	0.399±0.012
IFN-treated HL-60	0.443±0.016	0.403±0.017

Description of the cell lines and the experimental procedure are described in the Methods of chapter III. The TNF-sensitive line does not grow in the presence of TNF, whereas the resistant line (L929-R) grows unaffected regardless of the presence of TNF.

APPENDIX 5

Multiple washings after the 2-day IFN treatment shows no TNF (or any other factor) production and/or accumulation that could influence the up-regulation of CSF-1 receptor as assessed by binding and response experiments. The experimental details are provided in the Results of chapter III.

	Net binding*	^{3}H-TdR incorporation (± SD)
Untreated HL-60	574 ± 86	48346 ± 1023
CSF-1-treated HL-60	466 ± 112	45689 ± 2331
IFN-treated HL-60	4985 ± 277	35466 ± 1542
IFN-treated HL-60 (not washed)	7555 ± 399	64264 ± 1436#
IFN-treated HL-60 (washed 3X)	5987 ± 233	65375 ± 1295#

* Net binding represents the difference between the non-specific and total binding.

CSF-1 was added for the ^{3}H-TdR incorporation assay.

APPENDIX 6

HL-60 supernatants tested on a murine cell line co-culture of NOB-1 and CTLL show no production of IL-1. In this assay, NOB-1 cells produce IL-2 in response to IL-1, which in turn stimulates the growth of CTLL cells as assessed by ^{3}H-TdR incorporation.

	^{3}H-TdR incorporation (± SD) on:		
Factor or Sup.	NOB-1	CTLL	NOB-1+CTLL*
---	54 ± 12	350 ± 64	401 ± 103
IL-1 (50 U/ml)	48 ± 18	378 ± 82	12722 ± 980
Untreated HL-60	84 ± 32	422 ± 46	564 ± 124
IFN-treated HL-60	75 ± 15	531 ± 79	628 ± 144

* The culture conditions as well as the experimental protocol for this experiment are described in detail in the Methods of chapter III. The results reported here represent two experiments carried out in triplicates.

APPENDIX 7

IFN and IL-1 do not synergize to up-regulate CSF-1 responsiveness of HL-60 cells.

Treatment*			^{3}H-TdR incorporation
IFN (U/ml)	IL-1 (ng/ml)	CSF-1 (U/ml)	(cpm ± SD) on day 10
--	--	--	51443 ± 1399
100	--	--	39989 ± 929
10	--	--	41221 ± 1355
1	--	--	48176 ± 888
100	--	1000	56286 ± 549
10	--	1000	44233 ± 2467
1	--	1000	44756 ± 1144
--	0.2	--	68219 ± 775
--	--	1000	52378 ± 1644
--	0.2	1000	65333 ± 1321
100	0.2	--	44190 ± 3212
10	0.2	--	61396 ± 2565
1	0.2	--	66878 ± 2178
100	0.2	1000	65561 ± 2443
10	0.2	1000	68009 ± 3036
1	0.2	1000	59932 ± 2978

* These results represent two experiments that were carried out in triplicates.